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PRINCIPAL INVESTIGATION: Claudius Vincenz, Ph.D.

CONTRACTING ORGANIZATION: University of Michigan
Ann Arbor, Michigan 48103-1274

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Ann Arbor, Michigan 48103-1274

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Abstract

Genetic experiments in C. elegans have shown that a triad of genes consisting of CED3, CED4, and CED9 is necessary for all 131 apoptotic events occurring during the development of the nematode. Homologues of these genes have been identified in mammals as the caspase family, Apaf-1, and the Bcl-2 family. Targeted gene studies have shown that most caspases involved in apoptosis have a non redundant function. Apaf-1 also seems to be involved in most apoptotic events. On the other hand, the mild phenotypes of the knock out mice for Bcl-2 family members, suggest redundancy within this family. We have characterized a novel Bcl-2 homologue, Boo, that is only expressed in the ovaries and epididymis. This tissue specific expression suggests that in mammals the central C. elegans triad has been modified. Particularly the Bcl-2 family has been varied to create tissue specific death effector pathways. It will be interesting to explore the pharmacological possibilities that must exist as a result of this tissue specificity. We have also further characterized caspase-14 by in situ hybridizations and show that it is expressed in areas of the brain and thymus with high levels of apoptosis. Caspase-14 is only expressed during mouse development which suggests that caspase-14 is involved in apoptosis during development. We have used conditionally transformed striatal cells to study apoptosis in neuronal systems. Using this system we discovered that wild type Huntingtin protects from multiple apoptotic inducers strongly suggesting that it inhibits the activity or the activation of Caspase-9. We are currently exploiting this system for other neuron specific apoptotic events such as p75 NGF signaling.

FOREWORD

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Table of contents

Introduction:

The view that apoptosis is a central process regulating tissue homeostasis during development and in pathological conditions was further corroborated during the last year. Particularly the targeted gene experiments with Apaf-1 (Cecconi et al., 1998; Yoshida et al., 1998) Caspase-9 (Hakem et al., 1998; Kuida et al., 1998), and Caspase-8 (Varfolomeev et al., 1998) have shown how central these molecules are for the process. Some of these molecules were initially described using funding from this grant.

An important role for dysregualtion of apoptosis in cancer has been well established for many years. During the last year some key publications were able to show that excessive apoptosis occurs during neurodegenerative diseases. While excessive apoptosis during neurodegenerative diseases had been postulated for many years, hard proof of this postulate had been lacking (Saudou et al., 1998).

The initial focus of the lab was on identifying components of the cell death pathway involved in death receptor signaling. This effort led to the discovery of some of the key molecules involved in apoptosis such as Caspase-3, FADD, Caspase-9, Caspase-8, Caspase-10 (Chinnaiyan et al., 1995; Muzio et al., 1996; Tewari et al., 1995; Vincenz and Dixit, 1997).

We have expanded our scope in subsequent studies to include mechanistic investigations on how the apical caspase-8 is activated and regulated (Hu et al., 1997; Muzio et al., 1998). We have also shown evidence that the *Caenorhabditis elegans* paradigm of cell death is conserved in mammals. In *C. elegans* Ced-3 interacts directly with Ced-4 and the anti-apoptotic protein Ced-9 which is part of this complex controls the activity of this apaptosome. Our results show that a homologous complex can be formed in mammals between Caspase-9, Apaf-1, and $BclX_L$ [Pan, 1998 #938].

In this report we identify yet another member of the Bcl-2 family, Boo. This protein is only expressed in the ovaries and epididymis. It inhibits apoptosis by growth factor withdrawal, γ-radiation., and cyclosporin. It also inhibits apoptosis induced by expression of the pro-apoptotic Bcl-2 family members Bik and Bak.

We continue the characterization of Caspase-14, a developmentally regulated caspase. We show by in situ hybridization that its expression during development correlates strongly with areas where apoptosis occurs.

In my search for a neuronal system to study apoptosis we started a collaboration with Elena Cattaneo from the University of Milano in Italy. We used conditionally transformed striatal cell lines to study the effect of wild type and mutant Huntingtin. This study for the first time revealed a function for the wild type protein. We plan to use this system to study other neuron specific apoptotic events like P75NTR killing.

During this year the lab was successful in moving from identifying steps that are important for all apoptotic events to looking at events that are either tissue specific (ovaries, neurons) or occur only during a restricted time period of the life of the organism (embryonic development). Likely, these specific events are the best pharmacological targets as they allow interference with the apoptotic process without global side effects.

Body:

1) Identification of a novel negative regulator of cell death SOW 2c,d

In an effort to identify additional molecules involved in the regulation of apoptosis we screened the public EST database with various Bcl-2 molecules. Using the chicken Bcl-2 homologue NR13 we were able to identify a mouse homologue. We named this homologue Boo for Bcl-2 homologue of ovaries.

A detailed description and characterization of this protein is presented in the appended publication (Song et al., 1999). This includes the methods section. I will therefore limit the discussion to the important points and unpublished results.

Clearly Boo is a member of the Bcl-2 family of proteins as it contains most of the conserved domains. BH4, BH2, BH1, and a transmembrane domain (TM) can be identified. The only domain that is poorly conserved is the BH3 domain.

Boo is exclusively expressed in the ovaries and epididymis. This limited tissue expression of a Bcl-2 homologue is highly unusual. Most Bcl-2 family members have a wide tissue expression, with the exception of Bok, a pro-apoptotic Bcl-2 homologue that is also expressed only in reproductive tissues (granulosa cells, testis and uterus) (Hsu et al., 1997). These "specialized" Bcl-2 homologues may point to tissue specific mechanisms of apoptosis. The prospect of an ovarian specific apoptotic mechanism would be a very significant finding as it would open up the possibility to interfere with the process pharmacologically. Ovarian apoptosis has been implicated in ovarian atresia and ovarian cancers are correlated with lack of apoptosis (Tilly, 1996).

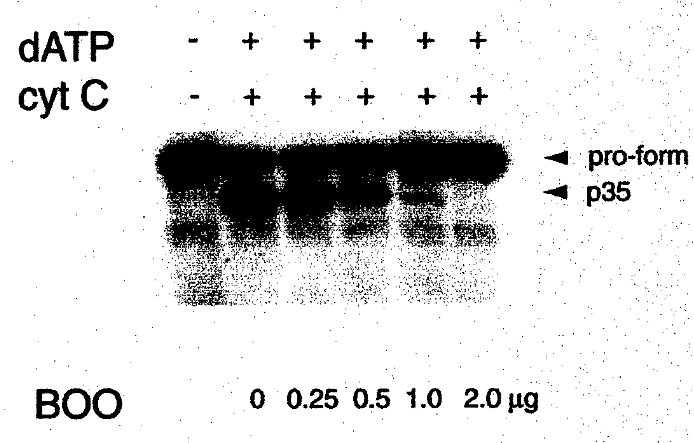
Boo is an anti-apoptotic molecule protecting cells from the following stimuli: Growth factor withdrawal, γ-irradiation, and cylcosporin A. These anti-apoptotic activities are similar to the well established protectors Bcl-2 and $BclX_L$. Boo also protects from apoptosis induced by pro-apoptotic Bcl-2 family members. Significantly, Boo only protects against Bik and Bak, the only members of the pro-apoptotic group with which Boo is able to interact. This observation lends support to the idea that the inhibitory function is requires heterodimerization with a pro-apoptotic Bcl-2 homologue.

We identified Apaf-1 as potential molecular target for Boo during co-immunoprecipitation. These observations are very similar to what the lab had previously described for $BclX_L$ (Pan et al., 1998). In this paper we extended the observation beyond showing that Boo can form a ternary complex with Apaf-1 and Caspase-9. We show that three domains of Apaf-1 can interact with Boo. Deletion constructs containing just the CED4, Apaf-1, or the WD-40 domain of Apaf-1 are all able to bind Boo. Boo is the second anti-apoptotic Bcl-2 homologue that is able to bind Apaf-1, further confirming that the apoptotic triad between Ced3,Ced4, and Ced9 described in C. elegans is conserved in mammals.

Since the publication of the article we have made a bacterial expression construct that expresses $(His)_6$ Boo in bacteria. We have used this recombinant protein in *in vitro* assays to measure its anti-apoptotic function.

Figure 1

Purified BOO inhibits Apaf-1-dependent maturation of caspase-9 in vitro



Boo inhibits cytochrome c induced activation of Caspase-9 by Apaf-1 in vitro.

20 µg of 293T S100 exctract proteins were incubated with 1 µl of in vitro transcription translated Caspase-9. ATP (1 mM), cytochrome c (0.4 µg), and Boo were added as indicated. The final reaction volume was 25 µl. Incubations were 30 min at 37 °C. Analysis was performed by SDS PAGE and Fluorography.

Methods: S100 cell extracts from 293 cells were prepared as follows. Cells were harvested in PBS, washed twice in cold PBS, followed by swelling in Buffer A (20 mM Hepes-KOH [pH 7.5], 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol [DTT], and 0.1 mM PMSF) and homogenization through a 22Gauge needle. The cleared lysates were further fractionated by centrifugation at 100,000 g in a table top ultracentrifuge. The supernatant was aliquoted and frozen at –80 °C.

Caspase-9 was in vitro transcription translated with a commercially available kit following the manufacturers instruction (Promega). Cytochrome c was purchased from Sigma.

A Boo expression construct without the transmembrane domain was engineered in Pet23b (Novagen) with a C terminal $(His)_6$ tag. Recombinant protein was expressed in BL21 cells and purified with a Ni^{2+} resin. The purity of the protein was > 95%.

The results of this experiment clearly show the ability of recombinant Boo to inhibit Apaf-1 mediated Caspase-9 activation. We therefore have been able to show the anti-apoptotic function of Boo in stable cell lines, transient assays, and *in vitro*.. This evidence is in our view convincing and refutes claims to the contrary by the Nunez group (Inohara et al., 1998).

2) Characterization of Caspase-14
SOW 2d

As mentioned in last years report we identified a new caspase, Caspase-14. This work has been published in the meantime and a copy is attached (Hu et al., 1998). Therefore again I report here only the important points and discuss in detail only the unpublished results.

Caspase-14 was identified by its homology in the catalytic domain with the other caspases. Its most notable feature is the complete lack of a pro-domain, which raises the question of how the activity of this caspase is regulated. All other caspases are synthesized as an inactive zymogen that is proteolytically activated by cleavage of the pro-domain. The possibility that Caspase-14 is regulated on the transcriptional level is raised by the observation that this caspase is only expressed during development. Two observations indicate that Caspase-14 is involved in apoptosis. (i) Transient overexpression of the wild type enzyme in MCF7 cells causes apoptosis while the active site mutant Casp-14 does not. The recombinant protein, expressed in bacteria and purified through a two column procedure, cleaves the Caspase-3 substrate Ac-DEVD-Afc *in vitro*.

To further establish a link between Caspase-14 expression and apoptosis we performed *in situ* hybridizations on developing mouse embryos. Comparisons of sagittal slices with sense ant antisense digoxygenin labeled riboprobe revealed high expression in several regions of the brain and thymus. Highest levels of Caspase-14 mRNA are found in the posterior base of the neopalllial cortex. Moderate levels of MICE mRNA are found in the intermediate zone of the brain. The developing central nervous system has been shown to undergo massive apoptosis and these results suggest that MICE could play a role in programmed cell death of neuronal cells in the CNS.

Figure 2

Caspase-14 mRNA Expression in the Developing Brain

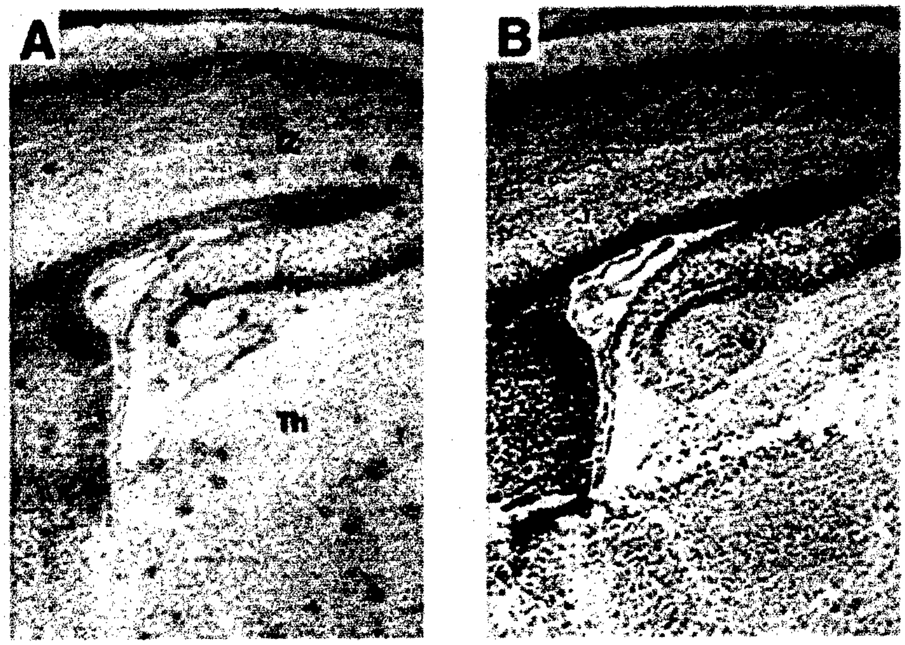


Figure 4- Sagittal brain section at 5X magnification probed with sense, A and antisense caspase-14 probe, B. Caspase-14 mRNA is widely expressed throughout the developing brain. (cortex, C; intermediate zone, IZ; ventricular zone, VZ; hippocampus, Hp; thalamus, Th)

Figure legend

Methods

In situ hybridization reagents and polymerase chain reaction (PCR) reagents were purchased from Boehringer Mannheim Biochemicals (BMB). PCR was performed using the Expand High Fidelity PCR System. 10 _m midsagittal frozen E17.5 CD1 mouse embryo sections were obtained from the University of Michigan Morphology Core. Sense and antisense caspase-14 probes containing the T7 RNA promoter sequence at the 5' end were synthesized (sense primer pair: 5'CGGAATTCTAATACGACTCACTAGTGCGCA TGTTCCGTTACCTGAAATTTGAAAGC3'/ 5'TTCCTCATTTCCACGTAGTTCCTCACCGGG3' and antisense primer pair: 5'GCCTTAAGATTATGCTGAGTGATCACTTCCTCATTTCCACGTAGTTCCTCACCGG G3'/ 5'CGCATGTTCCGTTACCTGAAATTTGAAAGC3') corresponding to amino acids 44-152 of Caspase-14. PCR products were gel purified and equimolar amounts of DNA was used for *in vitro* transcription with T7 RNA polymerase. DIG-labeled probes were generated using the DIG RNA labeling kit from BMB. Equimolar sense and antisense probe concentrations were determined by dotblotting serial dilutions of each probe onto nylon membranes followed by anti-digoxigenin and BCIP/NBT treatment. Non-radioactive *in situ* hybridization using dUTP-digoxigenin labeled sense and antisense probes was performed according to the protocol described in the Boehringer Mannheim non-radioactive *in situ* hybridization manual.

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3) Development of a neuronal system to study apoptosis SOW 2b

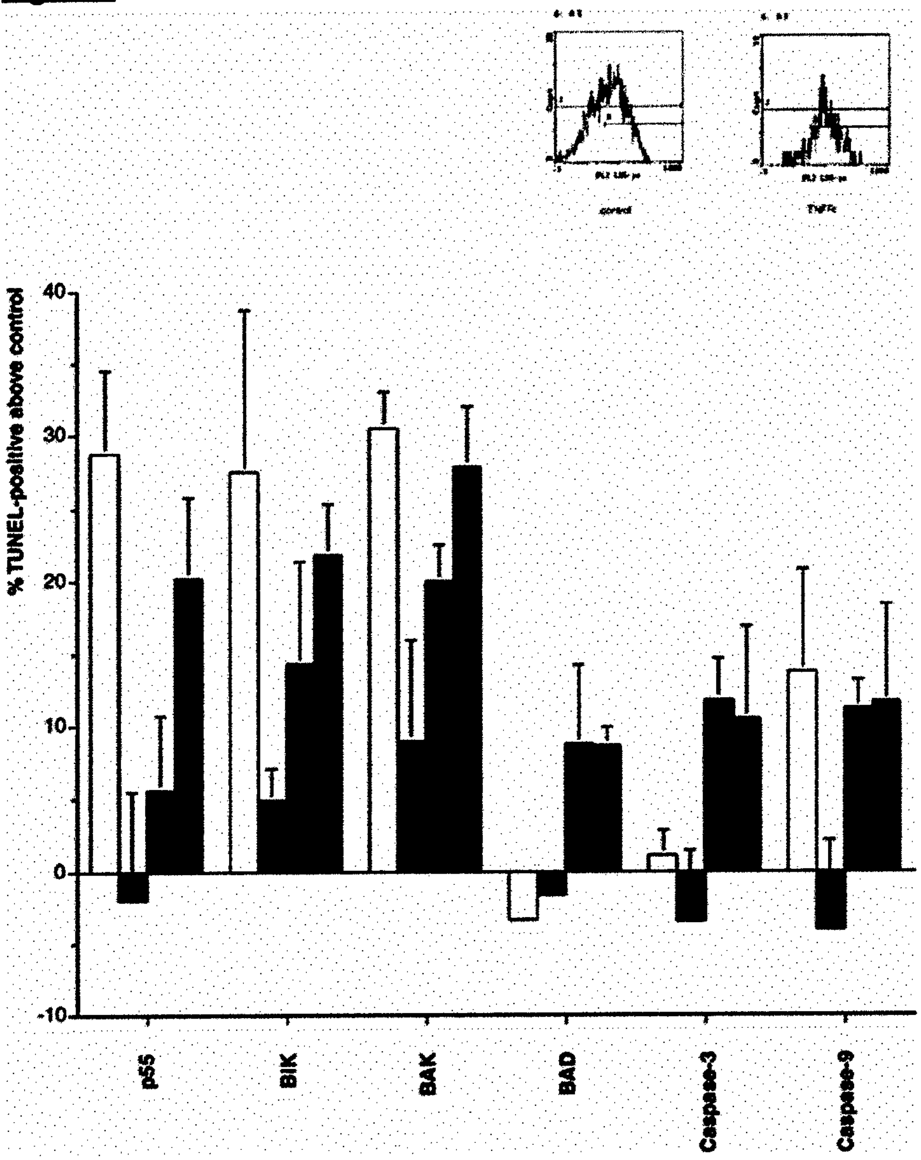
While it is well established that apoptosis plays an integral part in shaping the nervous system during development, *in vitro* systems that would allow the study of the process by biochemical techniques are lacking. In our attempt to identify such a system we established a collaboration with Elena Cattaneo to use conditionally transformed ST14A cells (Cattaneo and Conti, 1998). As Dr. Cattaneo has an interest in Huntingtins disease (HD) we developed the system to study apoptotic effects relating to HD. I will only give a detailed description of the experiments that were performed in my lab. Only a summary of the work done in Dr. Cattaneo's lab will be presented here.

ST14A cells are derived form the striatum of rat embryos. They have been immortalized by transfection with a temperature sensitive form of the SV-40 large T antigen. This systems makes it possible to expand and grow neuronal cells *in vitro* at the permissive temperature (33 ^{0}C). Shifting the temperature to 39 ^{0}C reverses the transformation and the cells start to differentiate. Studies have shown that ST14A cultured at the non-permissive temperature have maintained several properties of genuine striatal cells, like expression of striatal markers and the electrophysiological properties of neurons (Cattaneo and Conti, 1998)and Cattaneo pers. com.

Stable cell lines were established expressing full length and several truncation mutants of wild type and poly-Gln expanded huntingtin. Expression of the exogenous Huntingtin protein was verified by western and immunocytochemistry. These cell lines were initially exposed to a 39 ^{0}C in serum free medium. Under these conditions the parental cells dye by apoptosis in about 48 hrs. Lines expressing truncated mutant constructs were much more susceptible to these conditions and died by apoptosis earlier (12hrs). Most significantly was the fact that cells expressing the wild type version of Huntingtin did not dye up to 72 hrs after the shift in culture conditions. This protective effect was observed with full length and a construct truncated at amino acid 547. No protection was observed with a protein truncated at amino acid 63. Full length mutant protein was also protective from apoptosis induced by serum free culture at 39 ^{0}C.

The temperature shift in serum free media produces is an ill defined apoptotic stimulus. We therefore developed in my lab a transient transfection assay to express pro-apoptotic genes in ST14A cells. The assay is evaluated by two color FACS analysis after TUNEL labeling.

Figure 3



TUNEL of the stable cell lines with different death inducers

Parental cells (ST14A,white bars), wild type 1-547 Htt (black), mutant 1-547 Htt (light grey) and mutant full length expressing cells (dark grey) were transiently transfected with 1μg of the indicated plasmids. In ST14A cells TNFRI/p55, BIK, BAK and Caspase-9 induce apoptosis whereas BAD and Caspase-3 show no effect. Apoptosis by all inducers is blocked in wild type Htt expressing cells. Mutant Htt expressing cells show similar levels of apoptosis as the parental line for p55, BIK, BAK and Caspase-9 induced apoptosis and a marked increase in BAD and Caspase-3 induced apoptosis.

Shown is the average of at least two independent experiments; error bars show the average deviation. The inset depicts a typical TUNEL readout for TNFRI/p55. The control protein used is 14-3-3.

Methods

2-3x10^5 cells were transfected with 250ng pCMV-EGFP and 1μg of the indicated plasmids (0.4μg for Bik and Bak). After 24 hrs the cells and their supernatant were harvested and terminal transferase dUTP nick-end labeling (TUNEL) was essentially performed as described previously using PE-conjugated Avidin (BMB) (Gorczyca et al., 1993)). Stained cells were analyzed by dual color FACS. The number of TUNEL-positive cells was estimated by gating on the EGFP expressing cells. Background death as estimated by transfection of control protein 14-3-3 was subsequently subtracted from the data.

This analysis reveals that the protective effect of wild type Huntingtin is also observed if the cells are exposed to a variety of well defined death stimuli. Death receptor induced apoptosis is effectively inhibited by wild type Huntingtin. Similarly the pro-apoptotic Bcl-2 homologues Bik and Bak are only able to induce apoptotis effectively in parental cells and cells expressing mutant forms of Huntingtin. Mechanistically the most significant result is the fact that wild type Huntingtin also protects from Caspase-9 induced cell death. The effector arm of the apoptosis machinery is not affected by expression of Htt, because staurosporine is able to induce cell death in all cell lines equally well. These results identify Caspase-9 as the molecular target for the protective function of Huntingtin.

This is the first report of a function for wild type Huntingtin. This observation corroborates the knock out results which indicated that the gene was essential for survival of the embryos (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). We were able to map the survival function of Htt to the a amino terminal domain between amino acids 63 and 547. Further, these results are consistent with a gain of function event that affects only a subset of cells expressing Htt. These results are in contrast to reports claiming that the gain of function activity operates through the Caspase-8 pathway (Sanchez et al., 1999). The importance of using full length constructs to elucidate the gain of function mechanism is evidenced by the fact that the polyglutamine expansion in the context of the full length protein elicits different responses than the same mutation in a truncated construct. Our results indicate that the pro-apoptotic cytotoxicity generated by polyglutamine repeats involves a mechanism leading to caspase-3 activation. The upstream events leading to caspase activation are not fully understood at this point.

The results presented so far are being submitted to Nature medicine and should be considered confidential until acceptance of the paper.

We are continuing to investigate the inhibitory activity of Huntingtin on Caspase-9 activation. To this end we are making the appropriate reagents to study Caspase-9 activation *in vitro* with purified reagents.

We have also begun studies to exploit ST14A system to study apoptotic signaling from the p75NTR receptor. This receptor has been implicated by several studies to generate a pro-apoptotic signal (Casaccia-Bonnefil et al., 1996; Frade et al., 1996; Rabizadeh et al., 1993) . Surprisingly very little progress has been made in elucidating the signaling molecules involved in this process . This is in contrast to insight that has been obtained for the signaling of the other members of the TNFR, NGFR superfamily of receptors. Developing the ST14A cell system for this problem will open up new experimental approaches that were not available up to now using primary cell lines.

SOW 1d

We are developing a transgenic mouse model to study the importance of death receptors in the CNS during development and for the pathogenesis of neurodegenerative disorders.

To this end we have made a transgenic construct expressing FLAG tagged E8 under the control of the prion promoter. E8 is a gene product of the poxvirus and effectively inhibits apoptosis from all known death receptors (Hu et al., 1997; Thome et al., 1997). The prion promoter provides high level and tissue specific expression of the transgene (Borchelt et al., 1996; Fischer et al., 1996).

The expression of the construct was tested in N2A and 293 cells. A FLAG tagged protein of the right size was seen on western blots. We further developed two sets of primers and conditions that allow the identification of transgene incorporation at 1 copy / genome.

The construct is being microinjected and we expect the first mice within a month.

Assessment of activation status of long pro-domain caspases

SOW 2c

Antibodies for long pro-domain caspases have become commercially available. According to the original statement of work the intention was to use these antibodies to decipher which physiological apoptotic stimuli are activating which apical caspase. During the last year targeted disruptions of the apical caspases 8 and 9 have been reported (Hakem et al., 1998; Kuida et al., 1998) (Varfolomeev et al., 1998). These studies have clearly delineated the differential role for each of these caspases in different apoptotic processes. I have decided that repeating these studies with the antibodies would be redundant and inferior approach.

The only apical Caspase for which no knock out mouse exists is Caspase-10. We have used the antibody available from Millenium to study its processing. The first experiments were done in the Jurkat cell line and FAS activation by an agonistic antibody. A band at ~55 kd was clearly visible. Surprisingly this band was not processed upon FAS activation. This is unexpected as the characterization of Caspase-10 in transient assays implied the it was involved in FAS apoptotic signaling (Vincenz and Dixit, 1997). We are currently investigating if this discrepancy is due to non-specific antibody or artifacts of the overexpression system.

Two hybrid screen with DED containing protein
SOW 2a

We have analyzed the positive clones identified during a yeast two hybrid screen with the I-FLICE pro-domain containing two death effector motifs. We tested for the specificity of the interaction by cotransfection with different baits. Unfortunately no clone exclusively interacted with the I-Flice bait. In addition, sequence analysis of the clones revealed no death effector motifs of any other signature sequence known to be involved in apoptosis. At this point I decided to abandon this approach.

SOW 2c
No new death effector domain molecules were identified by screening the public EST databases. This is probably due to the fact that this approach is reaching saturation as most messages are represented in these databases.

Key Research accomplishments

- Cloning and characterization of Boo, a novel anti-apoptotic Bcl-2 homologue
- Characterization of Caspase-14
- Identification and characterization of the anti-apoptotic function of Huntingtin

Reportable outcomes

Publications

Hu, S., Snipas, S. J., Vincenz, C., Salvesen, G., and Dixit, V. M. (1998). Caspase-14 is a novel developmentally regulated protease. J Biol Chem *273*, 29648-53.

Song, Q., Kuang, Y. P., Dixit, V. M., and Vincenz, C. (1999). Boo, a novel negative regulator of cell death, interacts with Apaf-1. Embo J *18*, 167-178.

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Training

Shimin Hu: Graduate student department of pathology

Qhizong Song: Post-doctoral training

Conclusion

The identification and characterization of Boo, an anti-apoptotic Bcl-2 homologue, revealed that proteins believed to be central to the control of apoptosis can have very limited tissue expression. Boo is only expressed in the ovaries on epididymis. It suggests that the apoptotic machinery shows tissue specific modifications in its composition and presumably its function. Most significant is the fact that this tissue specificity is observed with a Bcl-2 homologue. A family of proteins involved in the control of the central apoptotic machinery and not in signal transduction. Therefore, components of the central apoptotic machinery can be feasible drug targets to achieve tissue specific effects.

We have further characterized caspase-14 and obtained more evidence for its involvement in apoptosis. In situ hybridization revealed that Caspase-14 is highly expressed in the CNS and thymus. Extensive apoptosis occurs in these organs during development. Gene targeting experiments will be performed to establish if Caspase-14 plays a non-redundant role in apoptosis during development.

Apoptosis is the CNS is known to be important during development and several neurodegenrative diseases. The experimental tools available for cell biological studies in neuronal systems are much more limited than for example in haematopoietic systems. We used conditionally transformed ST14A cells as a new tool to investigate apoptosis in neuronal systems. In a first application we studied the effect of Huntingtin in this system. We were able for the first time to show that wild type Huntingtin has a role in protecting cells from apoptosis. By taking advantage of the transfectability of ST14A cells we identified the molecular target of Htt to be caspase-9. We also observed pro-apoptotic effects elicited by mutant Huntingtin. The polyglutamine expansion in mutant Huntingtin induces Caspase-3 processing activity. The upstream events leading to this activation are currently not known but we expect to make fast progress on that front given the advantages of the ST14A system.

We are also exploiting the ST14A system to study apoptotic signaling from the p75 NGF receptor. Preliminary results show that p75 induces cell death in this system. To assess more broadly the function of death receptors in neuronal systems we are establishing a transgenic line that expresses the cow pox virus protein E8 under the control of the prion promoter. Because E8 inhibits apoptotic signaling from all known death receptors the phenotype of these mice should reveal the importance of death receptors during development.

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Appendices

Reprints

Hu, S., Snipas, S. J., Vincenz, C., Salvesen, G., and Dixit, V. M. (1998). Caspase-14 is a novel developmentally regulated protease. J Biol Chem *273*, 29648-53.

Song, Q., Kuang, Y. P., Dixit, V. M., and Vincenz, C. (1999). Boo, a novel negative regulator of cell death, interacts with Apaf-1. Embo J *18*, 167-178.

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Caspase-14 Is a Novel Developmentally Regulated Protease*

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Shimin Hu‡, Scott J. Snipas§, Claudius Vincenz‡, Guy Salvesen§, and Vishva M. Dixit‡¶‖

From the ‡Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109, the ¶Department of Molecular Oncology, Genentech, Inc., South San Francisco, California 94080, and the §Burnham Institute, La Jolla, California 92037

Caspases are a family of cysteine proteases related to interleukin-1 converting enzyme (ICE) and represent the effector arm of the cell death pathway. The zymogen form of all caspases is composed of a prodomain plus large and small catalytic subunits. Herein we report the characterization of a novel caspase, MICE (for mini-ICE), also designated caspase-14, that possesses an unusually short prodomain and is highly expressed in embryonic tissues but absent from all adult tissues examined. In contrast to the other short prodomain caspases (caspase-3, caspase-6, and caspase-7), MICE preferentially associates with large prodomain caspases, including caspase-1, caspase-2, caspase-4, caspase-8, and caspase-10. Also unlike the other short prodomain caspases, MICE was not processed by multiple death stimuli including activation of members of the tumor necrosis factor receptor family and expression of proapoptotic members of the bcl-2 family. Surprisingly, however, overexpression of MICE itself induced apoptosis in MCF7 human breast cancer cells, which was attenuated by traditional caspase inhibitors.

Major advances have been made toward understanding the molecular mechanism of programmed cell death (1). Functioning as central components of the cell death signaling pathway are a rapidly growing family of cysteine proteases that cleave following aspartate residues (caspases)[1] (2, 3). Caspases are normally present as single polypeptide zymogens and contain an N-terminal prodomain and large (p20) and small (p10) catalytic subunits (4–6). The 2-chain active enzyme is obtained following proteolytic processing at internal Asp residues (4–6). As such, caspases are capable of activating each other in a manner analogous to the processing of zymogens observed in the coagulation cascade.

To date, twelve caspases have been identified that can be classified into three subfamilies: caspase-1 (interleukin-1 converting enzyme), caspase-4 (ICErelII, TX, ICH2), caspase-5 (ICErelIII, TY), caspase-11 (Ich-3), and caspase-12 belong to the caspase-1 subfamily; caspase-2 (Ich-1) is the sole member of the caspase-2 subfamily; caspase-8 (FLICE, MACH, Mch5), caspase-9 (ICE-LAP6, Mch6), caspase-10 (FLICE2, Mch4), caspase-3 (Yama, CPP32, apopain), caspase-7 (ICE-LAP3, Mch3, CMH-1), and caspase-6 (Mch2) belong to the caspase-3 subfamily (2, 3, 7). An alternate classification is based on the size of the prodomain because large prodomain caspases function as upstream signal transducers, whereas short prodomain caspases function as downstream amplifiers that cleave death substrates (8). It is not entirely clear how large prodomain caspases are activated; however, recent studies suggest that their binding to receptor-associated adaptor molecules results in their approximation and activation by autoprocessing (8–12).

Three short prodomain caspases exist in the caspase-3 subfamily, whereas none have been found in the other two subfamilies (2, 3). Here we report a novel developmentally regulated short prodomain caspase designated MICE or caspase-14 that is a member of the caspase-1 subfamily and possesses unique biochemical properties.

MATERIALS AND METHODS

Cell Lines and Expression Vectors—Human embryonic kidney 293 and 293-EBNA cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, nonessential amino acids, L-glutamine, and penicillin/streptomycin. Human breast carcinoma MCF7 cells were maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, nonessential amino acids, L-glutamine, and penicillin/streptomycin. Expression constructs of tumor necrosis factor receptor family members were in pFLAG-CMV-1 (Kodak). Bax, Bak, and Bik expression constructs were generously provided by G. Chinnadurai, IAP1 and IAP2 by D. V. Goeddel, and Hrk by G. Nunez. All other expression constructs were made in pcDNA3 (Invitrogen). Epitope tags were placed at the C termini unless otherwise indicated.

Cloning of Caspase-14 (MICE)—cDNA sequences corresponding to the partial open reading frame of caspase-14 were identified as expressed sequence tags (EST) (GenBank™ accession numbers AA103647 and AA167930) homologous to caspase family members. Both clones were sequenced using plasmid DNA template by the dideoxy chain termination method employing modified T7 DNA polymerase (Sequenase, United States Biochemical). The AU1 epitope-tagged version of MICE was obtained by polymerase chain reaction using custom synthesized primers.

Northern Blotting—Mouse adult multiple tissue and embryo tissue poly (A)+ RNA blots were obtained from CLONTECH and processed according to manufacturer instructions. A ^{32}P-labeled cDNA corresponding to MICE amino acid residues 44–152 was used as probe.

Transfection, Coimmunoprecipitation, and Western Analysis—Transient transfections of 293 cells were performed as described previously (13). Cells were harvested 20–30 h following transfection and either immunoprecipitated and immunoblotted or directly immunoblotted with the indicated antibodies.

Cell Death Assay—293 EBNA cells and MCF7 cells were transiently transfected with 0.1 and 0.25 μg of the reporter plasmid pCMV β-galactosidase, respectively, plus 0.5–1.0 μg of test plasmids in the presence or absence of 2.0 μg of inhibitory plasmids. 24–30 h following transfection, cells were fixed with 0.5% glutaraldehyde and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside. Percentage of apoptotic cells was determined by calculating the fraction of membrane-blebbed blue cells as a function of total blue cells. All assays were

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‖ To whom correspondence should be addressed: Dept. of Molecular Oncology, Genentech, Inc., 1 DNA Way, Bldg. 10, Rm. 290, South San Francisco, CA 94080. Tel.: 650-225-1312; Fax: 650-225-6127; E-mail: dixit@gene.com.

[1] The abbreviations used are: caspase, cysteine aspartate specific protease; ICE, interleukine-1 converting enzyme; MICE, mini-ICE; I-FLICE, inhibitor of Fas-associated death domain protein (FADD)-like ICE; TNFR1, tumor necrosis factor receptor 1; IAP, inhibitor of apoptosis; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

A

MESEMSDPQPLQEERYDMSGARLALTLCVTKAREGSEVDMEALERMFRYLKFESTMKRDPTAQQFLEELD
EFQQTIDNWEEPVSCAFVVLMAHGEEGLLKGEDEKMVRLEDLFEVLNNKNCKALRGKPKVYIIQACRGEH
RDPGEELRGNEELGGDEELGGDEVAVLKNNPQSIPTYTDTLHIYSTVEGYLSYRHDEKGSGFIQTLTDVF
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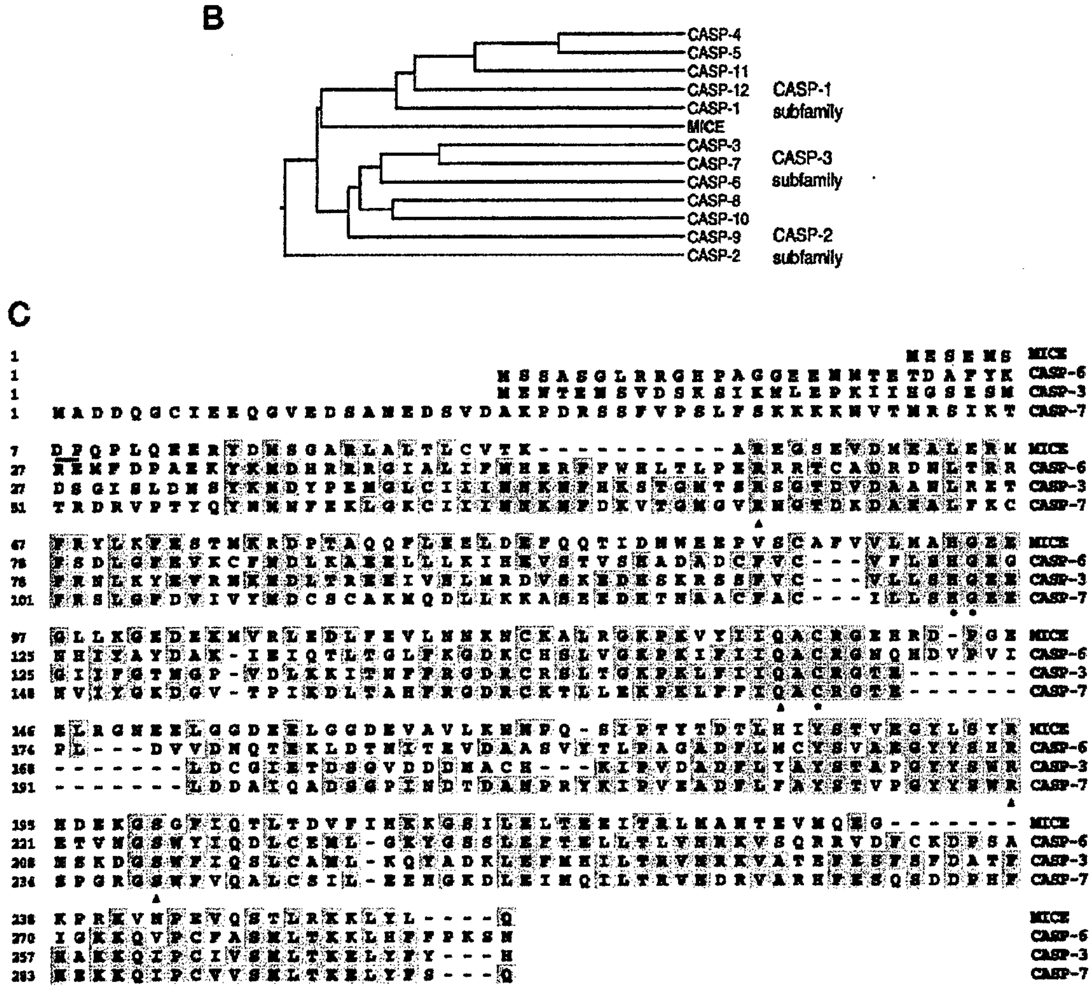

FIG. 1. **Sequence analysis of MICE.** *A*, deduced amino acid sequence of MICE. The conserved pentapeptide QACRG is *boxed*, and the putative cleavage site between prodomain and p20 and that between p20 and p10 are *underlined*. *B*, phylogenetic analysis of caspase family. *C*, sequence alignment of MICE and three known short prodomain caspases. *Solid circles* indicate residues involved in catalysis, and *triangles* identify residues that form the binding pocket for the carboxylate side chain of P1 Asp. The putative cleavage sites between prodomain and p20 are *underlined*. *CASP-*, caspase.

evaluated in duplicate, and the mean and standard deviation was calculated.

Mice Expression and Purification—Recombinant MICE was expressed in *Escherichia coli* strain Bl21 (DE3) plysS following induction for 4 h at 37 °C with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested by centrifugation, resuspended in 100 mM Tris-HCl, 100 mM NaCl, pH 8, and lysed by freeze-thaw cycles followed by sonication. The supernatant was recovered by centrifugation and applied directly to immobilized Ni-nitrilotriacetic acid for purification utilizing the engineered N-terminal His tag. The protein was eluted with a 0–200 mM imidazole gradient, and the recovered MICE was contaminated with an equal amount of *E. coli* histidine-rich protein. Final purification of MICE was achieved by ion exchange utilizing a gradient of 0–500 mM NaCl in 20 mM Tris-HCl following adsorption to DEAE-Sepharose. Approximately 1 mg of MICE was obtained from 3 liters of *E. coli*, and the final concentration was 0.3 mg/ml.

Mice Activity Assay—Purified MICE (10 μl) was added to 40 μl of caspase assay buffer (20 mM PIPES, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, pH 7.2) in the absence (control) or presence of the following caspase inhibitors: 2 μM Z-VAD-FMK, or 5 μM CrmA(REF) or 0.3 μM p35, followed by incubation at 37 °C for 30 min to allow for complex formation. Residual activity was assayed by adding 50 μl of a 0.2 mM solution of the caspase substrate Ac-DEVD-AFC, and measurement of released AFC at 37 °C using a Perkin-Elmer LS50 spectrofluorimeter with excitation at 400 nm and emission at 505 nm.

RESULTS AND DISCUSSION

MICE Is a Short Prodomain Caspase of Caspase-1 Subfamily—Analysis of the full-length cDNA sequence of MICE revealed a 774-base pair open reading frame that encoded a novel protein of 257 amino acids with a predicted molecular mass of 29.5 kDa (Fig. 1*A*). Comparison of this protein with all known caspases revealed that it had a unusually short prodomain of only six amino acids (Fig. 1, *A* and *C*). Given this, the molecule was termed MICE (for mini-ICE). The caspase designation for it is caspase-14.

Phylogenetic analysis revealed MICE to be most related to caspase-1 subfamily members, and it is therefore the first short prodomain caspase to be part of the caspase-1 subfamily (Fig. 1*B*). Overall, MICE displayed 21.4, 19.5, and 20.2% identity to

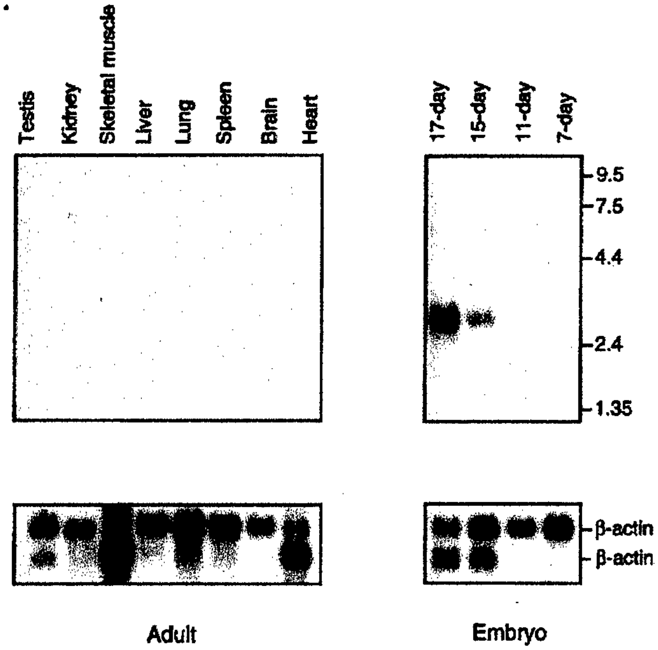

FIG. 2. **Tissue distribution of MICE.** Murine adult multiple and embryo tissue poly(A)$^+$ Northern blots were probed with ^{32}P-labeled MICE cDNA.

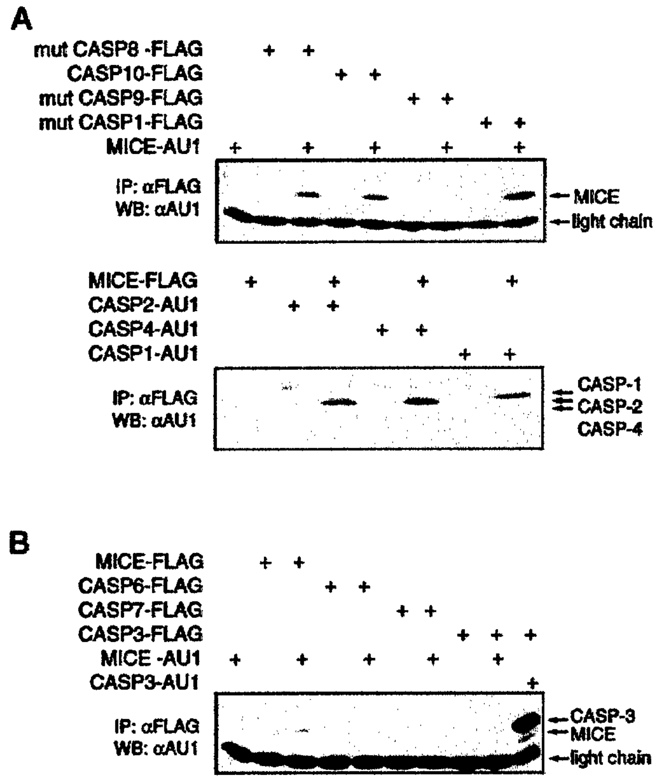

FIG. 3. **MICE preferentially associates with some large prodomain caspases.** 293 cells were co-transfected with the expression constructs encoding epitope-tagged MICE and other caspases. 30 h following transfection, cells were harvested, lysed, and analyzed with the indicated antibodies. The expression of each plasmid was confirmed by either reprobing the blots or directly blotting the cell lysate. *mut*, mutant; *CASP-*, caspase.

the known short prodomain caspases 3, 6, and 7, respectively (Fig. 1*C*). The QACRG pentapeptide motif present in most caspases is also conserved in this novel caspase. In addition, based on the x-ray crystal structure of caspase-1 and caspase-3, amino acid residues involved in catalysis are conserved in MICE as are residues that form a binding pocket for the carboxylate side chain of the P1 aspartic acid (Fig. 1*C*) (4–6). This is in keeping with MICE being a functional caspase.

Tissue Distribution of MICE—Mouse adult and embryonic tissue poly(A)$^+$ RNA blots were probed with a ^{32}P-labeled cDNA corresponding to the large catalytic subunit of MICE. A single transcript of 2.8 kilobases was observed (Fig. 2). Unlike almost all known caspases that are expressed in both adult and embryonic tissues (7, 10, 11, 14–18), MICE was highly expressed in certain stages of embryonic development but was undetectable in all adult tissues examined, including heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis. Interestingly, the expression level of MICE appeared to increase during the later stages of development (the attenuated expression in day 11 may be because of lower loading of mRNA).

MICE Preferentially Associates with Certain Large Prodomain Caspases—Because small prodomain caspases function downstream of large prodomain caspases, we asked if MICE, being a short prodomain caspase, bound any of the putative upstream large prodomain caspases. Surprisingly, MICE associated with most large prodomain caspases, including caspase-1, -2, -4, -8, and -10 (Fig. 3*A*). The other short prodomain caspases including caspase-3, -6, and -7 associated only with caspase-10 (data not shown). MICE did not bind the other short prodomain caspases and displayed only weak self-association in contrast to caspase-3 and other caspases that strongly self-associated (Fig. 3*B*, and unpublished data). The preferential dimerization with large prodomain caspases and weak self-association suggest that MICE may function through heterodimerization.

MICE Is Not Processed in Multiple Death Signaling Pathways—Previous studies have shown that initially long prodomain and then short pro-domain caspases are processed following activation of death receptors including TNFR1 and CD95 death receptors (19–24). To further characterize MICE, we asked if it was processed on activation of these receptors. 293 cells were transiently transfected with expression constructs encoding death signaling receptors and MICE, or the three known short prodomain caspases: caspase-3, -7, and -6. Interestingly, all three known short prodomain caspases were processed upon coexpression with the death signaling receptors (Fig. 4*A*). MICE, however, was not processed, suggesting that it is not involved in the death pathway engaged by these proapoptotic receptors (Fig. 4*A*).

Bax, Bak, Bik, Bad, Bid, and Hrk are proapoptotic members of the bcl-2 family (23, 25). 293 cells were transiently transfected with expression constructs encoding short prodomain caspases and proapoptotic bcl-2 family members. In keeping with the prior results, all three known short prodomain caspases were processed on co-expression, but MICE was not processed (Fig. 4*B*).

Because MICE was not processed on activation of a number of distinct physiologically relevant death pathways, we asked if it could serve as a substrate for known caspases. Expression constructs encoding MICE and known caspases were coexpressed in the presence or absence of the death signaling receptor TNFR1. Consistent with previous results, no processing of MICE was observed despite the additional death signal from TNFR1 (Fig. 4*C*). MICE was also not processed by caspase-1 or -4, both members of the caspase-1 subfamily (data not shown). The failure of processing of MICE suggests that it likely functions in a very specific pathway that remains to be defined.

MICE-induced Apoptosis Is Attenuated by Inhibitors of Apoptosis—To determine whether MICE plays a role in cell death, 293 EBNA and MCF7 cells were transfected with expression plasmids encoding wild-type MICE, a mutant version of MICE

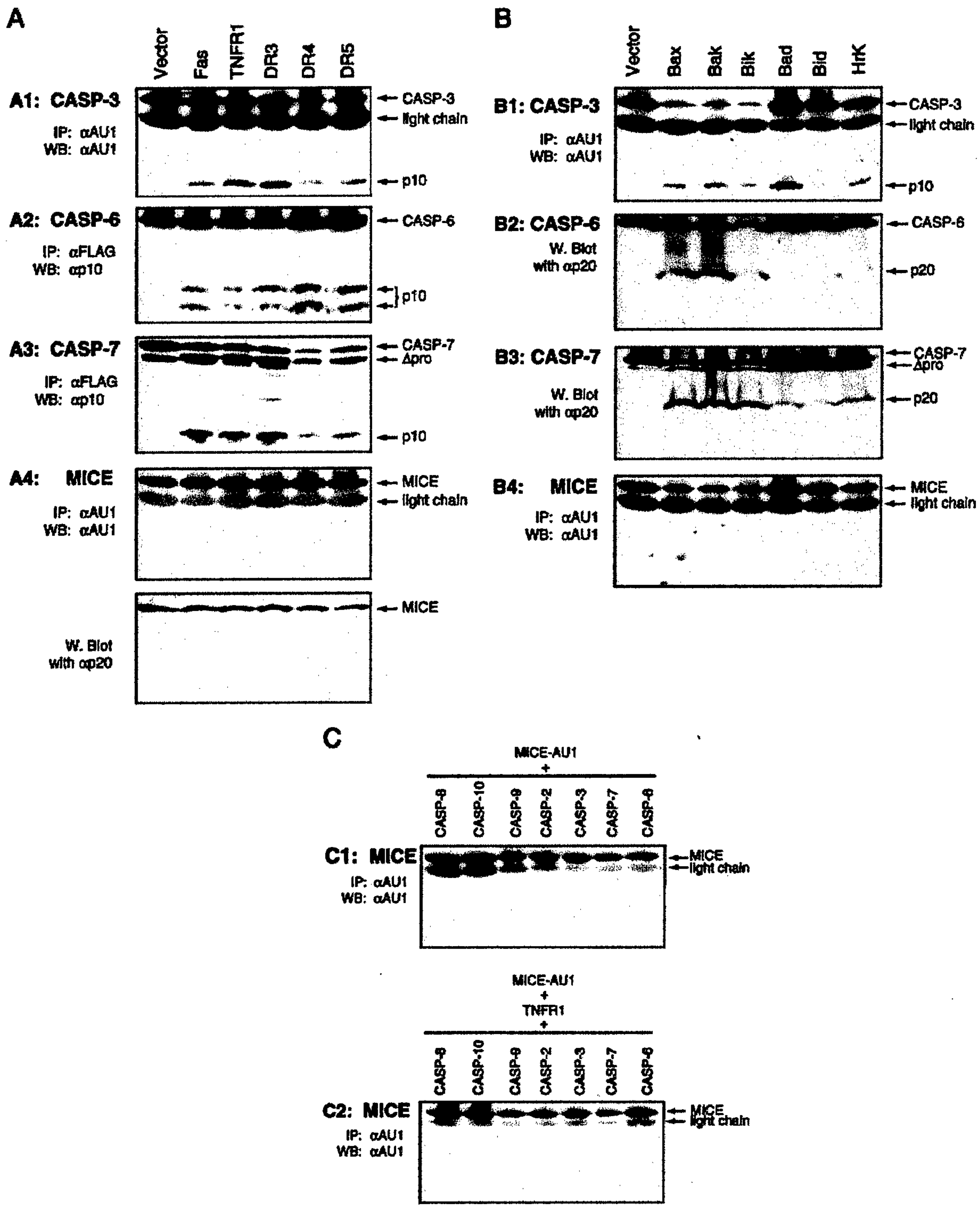

FIG. 4. **MICE is not processed on activation of known death signaling pathways.** 293 cells were co-transfected with expression constructs encoding TNFR family members and either C-terminal-tagged caspase-3-AU1, caspase-6-FLAG, caspase-7-FLAG, or MICE-AU1 (*A*). MICE was also co-expressed with the proapoptotic members of bcl-2 family (*B*) or caspases in the absence or presence of TNFR1 (*C*). 20–24 h following transfection, cells were harvested and either immunoprecipitated (*IP*) and immunoblotted or directly immunoblotted with the indicated antibodies. *WB*, Western blot; *CASP-*, caspase; *IP*, immunoprecipitated.

in which the presumed catalytic cysteine was altered to an alanine (QACRG to QAARG mutant) and caspase-8 as a positive control that has previously been shown to potently induce apoptosis in both cell lines (9, 10). Like the three other known short prodomain caspases, MICE had little effect on 293 EBNA cells (Fig. 5*A*, and unpublished data). However, it induced apoptosis in MCF7 cells (Fig. 5*A*). As expected, catalytically inactive MICE displayed substantially less death-inducing activity. More importantly, MICE-induced apoptosis in MCF7 was inhibited by the baculoviral-encoded inhibitors of apoptosis 1 and 2 (IAP1 and IAP2) and the broad spectrum baculoviral caspase inhibitor p35, but not by CrmA, MC159, or I-FLICE (Fig. 5*B*). CrmA is a cowpox serpin that inhibits caspase-1 and -8 activity, whereas MC159 is a death effector domain-containing decoy molecule encoded by molluscum contagiosum virus (26–27). I-FLICE is a naturally occurring catalytically inert dominant-negative caspase (28). These inhibitors function at the apex of the apoptotic cascade by disrupting assembly of

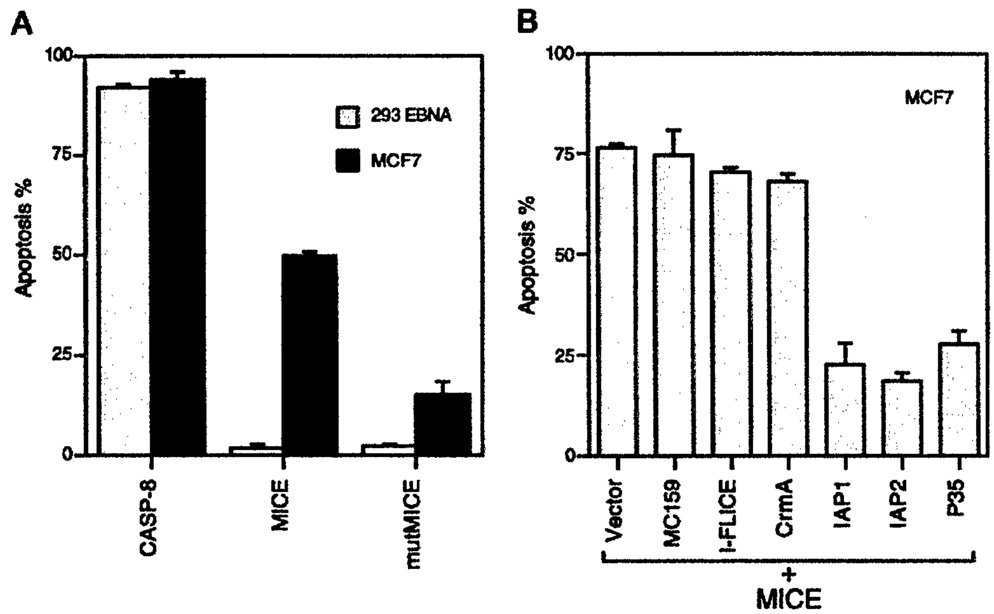

FIG. 5. **MICE-induced apoptosis is attenuated by the inhibitors of apoptosis.** *A*, 293 and MCF7 cells were co-transfected with a reporter gene (β-galactosidase) and expression constructs encoding caspase-8 (*CASP-8*), MICE, and a mutant version of MICE (*mutMICE*). *B*, MCF7 cells were co-transfected with the reporter gene and MICE expression construct in the absence or presence of the inhibitor plasmids. The cells were fixed and stained as described under "Materials and Methods." Expression of all transfected plasmids was verified by immunoblotting.

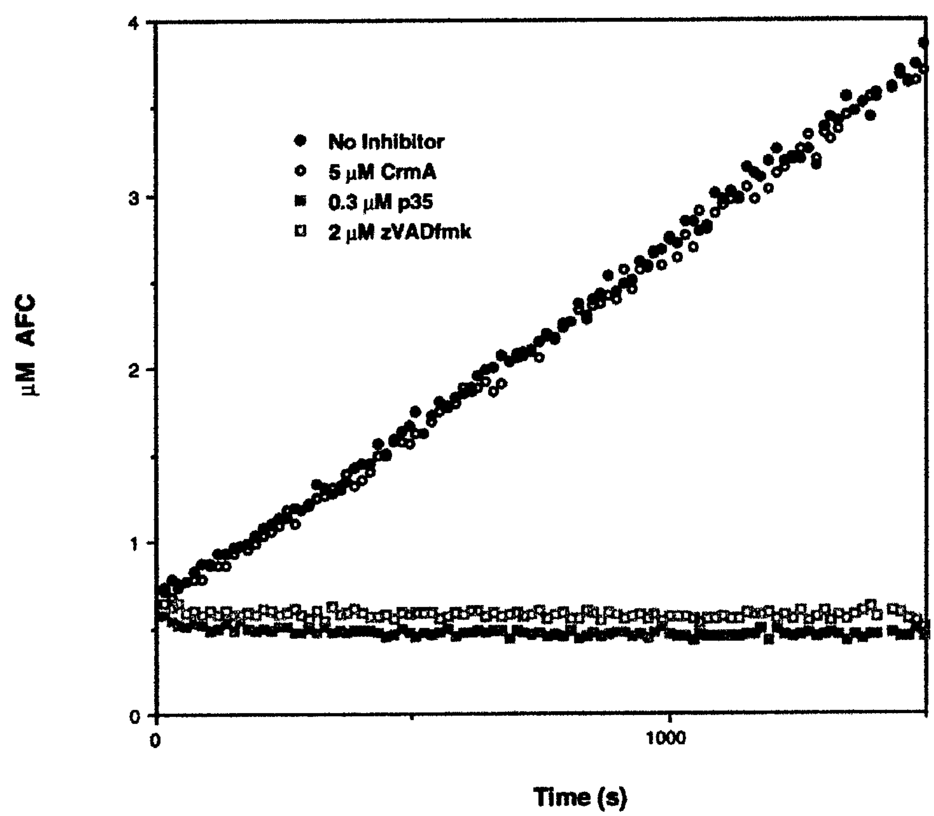

FIG. 6. **Characterization of MICE caspase activity.** The ability of recombinant MICE to cleave the fluorometric caspase substrate Ac-DEVD-AFc is shown as a function of the inhibitor present at time of initiation of assay.

receptor signaling complexes and/or inhibiting the initiating caspase (27–29). Because they had no effect on cell death induced by MICE, it is possible that MICE functions as a downstream signal transducer of cell death. However, it should be emphasized that the results from these overexpression studies should not be construed to imply a definitive role for caspase-14 in apoptosis. This will have to await more definitive studies including the generation of a mouse that is homozygous null for the gene in question.

MICE Possesses Caspase Activity—Recombinant MICE prepared by overexpression in *E. coli* possessed intrinsic caspase activity that was inhibitable by the broad spectrum caspase inhibitors zVADfmk and p35 but not by CrmA (Fig. 6).

In summary, the failure of MICE to undergo processing in multiple known death pathways and its ability to physically interact with large prodomain caspases and induce cell death suggests that MICE likely functions as a downstream active caspase in an as yet unidentified signaling pathway.

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Boo, a novel negative regulator of cell death, interacts with Apaf-1

Qizhong Song, Yongping Kuang, Vishva M.Dixit[1] and Claudius Vincenz[2]

Department of Pathology, The University of Michigan Medical School, Ann Arbor, MI 48109 and [1]Genentech, Inc., 1 DNA Way, M/S-40, South San Francisco, CA 94080, USA

[2]Corresponding author
e-mail: claudius.vincenz@umich.edu

In this report, we describe the cloning and characterization of Boo, a novel anti-apoptotic member of the Bcl-2 family. The expression of Boo was highly restricted to the ovary and epididymis implicating it in the control of ovarian atresia and sperm maturation. Boo contains the conserved BH1 and BH2 domains, but lacks the BH3 motif. Like Bcl-2, Boo possesses a hydrophobic C-terminus and localizes to intracellular membranes. Boo also has an N-terminal region with strong homology to the BH4 domain found to be important for the function of some anti-apoptotic Bcl-2 homologues. Chromosomal localization analysis assigned Boo to murine chromosome 9 at band d9. Boo inhibits apoptosis, homodimerizes or heterodimerizes with some death-promoting and -suppressing Bcl-2 family members. More importantly, Boo interacts with Apaf-1 and forms a multimeric protein complex with Apaf-1 and caspase-9. Bak and Bik, two pro-apoptotic homologues disrupt the association of Boo and Apaf-1. Furthermore, Boo binds to three distinct regions of Apaf-1. These results demonstrate the evolutionarily conserved nature of the mechanisms of apoptosis. Like Ced-9, the mammalian homologues Boo and Bcl-x_L interact with the human counterpart of Ced-4, Apaf-1, and thereby regulate apoptosis.

Keywords: Apaf-1/apoptosis/Boo/caspase-9/ovary

Introduction

Apoptosis, a morphologically distinct form of cell death, is important for normal development, tissue homeostasis and defense against pathogenic microorganisms (Raff, 1992; Vaux *et al.*, 1994). Perturbations of apoptosis can lead to a number of human pathologies such as cancer, auto-immune disease and neurodegenerative disorders (Thompson, 1995).

Despite the diversity of stimuli triggering apoptosis and the wide range of cell types involved, the apoptotic death machinery is evolutionarily conserved from nematodes to mammals. Genetic studies in the nematode *Caenorhabditis elegans* have identified three core components of the cell-death machine: *ced-3*, *ced-4* and *ced-9* (Hengartner and Horvitz, 1994c). *ced-3* and *ced-4* are required for the execution of the cell-death program and loss-of-function mutations in either gene result in the survival of all 131 somatic cells that normally die (Ellis and Horvitz, 1986). *ced-9*, in contrast, is a negative regulator of apoptosis and loss-of-function mutations in *ced-9* cause embryonic lethality as a consequence of ectopic cell death (Hengartner *et al.*, 1992). *ced-3* encodes a cysteine protease with aspartic acid specificity and is a relative of a family of caspases (Yuan *et al.*, 1993; Kumar and Harvey, 1995) that appear to act as the effectors of the cell-death pathway (Chinnaiyan and Dixit, 1996; Henkart, 1996). These death proteases exist as zymogens which are activated when the regulatory prodomain is removed and they assemble into active heteromeric proteases (Cohen, 1997). The caspase family now comprises 13 known members which can be divided into two classes based on the lengths of their N-terminal prodomains (Humke *et al.*, 1998). Accumulating evidence suggests that caspases with long domains, such as caspase-8, and -9, function upstream in the caspase cascade while caspases with short domains operate at the downstream end of the cascade (Cohen, 1997; Golstein, 1997). Activation of these distal caspases leads to proteolytic cleavage of a limited number of key protein substrates and execution of the death program (Cohen, 1997).

The death-repressor gene *ced-9*, like *ced-3*, also has multiple mammalian homologues and its protein product is structurally and functionally homologous to Bcl-2, the prototype member of a family of cell-death regulators (Hengartner and Horvitz, 1994b). First identified for its role in B-cell malignancies, Bcl-2 when overexpressed inhibits apoptotic cell death in diverse biological systems (Reed, 1994). Two functional classes of Bcl-2-related proteins constitute the family: anti-apoptotic members (Bcl-2, Bcl-x_L, Bcl-w, NR-13, A1 and Mcl-1), which inhibit cell death, and pro-apoptotic members (Bax, Bak, Bad, Bik, Bid, Hrk, Bim and Bok/Mtd), which promote apoptosis (Hsu *et al.*, 1997; Kroemer, 1997; Inohara *et al.*, 1998a; O'Connor *et al.*, 1998). Sequence alignments of Bcl-2 family proteins have identified four conserved domains, designated Bcl-2 homology regions, (BH1 to BH4) (Chittenden *et al.*, 1995a; Gibson *et al.*, 1996; Zha *et al.*, 1996). The BH1 and BH2 motifs of the death antagonists (such as Bcl-2 and Bcl-x_L) and the BH3 domain of the death agonists (such as Bax and Bak) are important for homo- or heterodimerization between family members (Yin *et al.*, 1994; Chittenden *et al.*, 1995b; Simonian *et al.*, 1996; Zha *et al.*, 1996). The BH4 domain, which is restricted to several anti-apoptotic homologues, appears to be essential for the death-repressing activity (Huang *et al.*, 1998). An early model proposed that the balance between pro- and anti-apoptotic Bcl-2 proteins determines cell fate (Oltvai *et al.*, 1993; Boise *et al.*, 1995; White, 1996; Kroemer, 1997). Contrary to this model, suppression or induction of cell death in some systems is independent of their interactions (Cheng *et al.*, 1996, 1997; Simonian *et al.*, 1996).

A

MADSQDPLHERTRRLLSDYIFFCAREPDTPEPPPTSVEAALLRSVTRQIQ
QEHQEFFSSFCESRGNRLELVKQMADKLLSKDQDFSWSQLVMLLAFAGTL
MNQGPYMAVKQKRDLGNRVIVTRDCCLIVNFLYNLLMGRRHRARLEALGG
WDGFCRFFKNPLPLGFWRRLLIQAFLSGFFATAIFFIWKRL

B

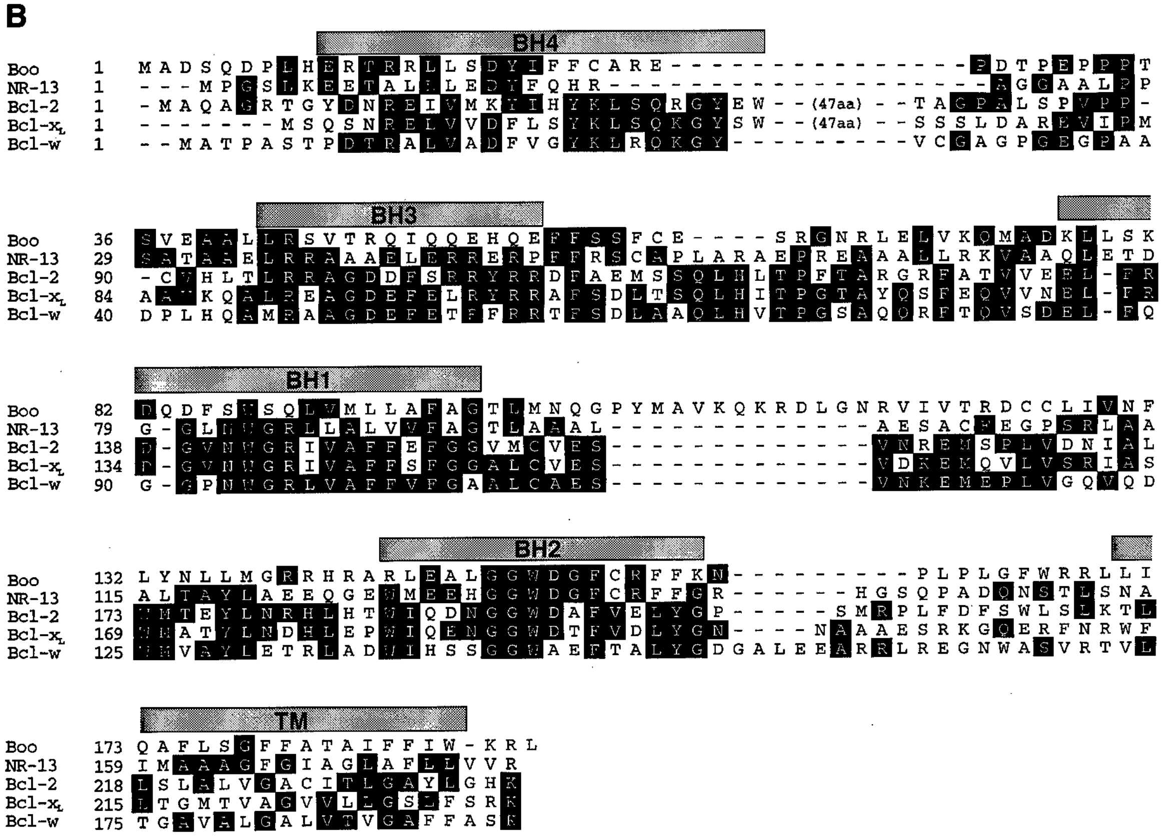

Fig. 1. Sequence characterization of Boo. (**A**) Deduced amino acid sequence of Boo. (**B**) Alignment of Boo amino acid sequence with those of mouse Bcl-2, Bcl-x_L, Bcl-w and chicken NR-13. The numbers on the left indicate the positions of the amino acid in each sequence. The conserved BH domains (1–4) and the C-terminal hydrophobic region (TM, transmembrane) are indicated. Shading marks identical residues. The DDBJ/EMBL/GenBank accession number for Boo is AF102501.

Genetic analysis in *C.elegans* indicates that Ced-9 prevents Ced-4 from activating the nematode caspase Ced-3 (Shaham and Horvitz, 1996a,b). Consistent with this, Bcl-2 and Bcl-x_L have been shown to function upstream of some of the caspases in the cell-death pathway (Armstrong *et al.*, 1996; Chinnaiyan *et al.*, 1996). However, the precise mechanism by which these death antagonists regulate caspase activation and apoptosis remains unclear.

Recently, a connection has been established between members of the Ced-9/Bcl-2 family and caspases. Ced-4 interacts with and promotes the activation of Ced-3, and this activation is inhibited by Ced-9 through direct interaction with Ced-4 (Chinnaiyan *et al.*, 1997; Spector *et al.*, 1997; Wu *et al.*, 1997a,b). Likewise, a similar mechanism also exists in mammalian cells. Apaf-1, a mammalian counterpart of Ced-4 (Zou *et al.*, 1997), simultaneously and independently binds to Bcl-x_L and caspase-9 (Hu *et al.*, 1998; Pan *et al.*, 1998). Bcl-x_L interacts with Apaf-1 and blocks Apaf-1-dependent caspase-9 activation (Hu *et al.*, 1998), further confirming the high degree of evolutionary conservation in the cell-death machinery.

In the present study, we report the identification and characterization of Boo, for Bcl-2 homologue of ovary, a novel anti-apoptotic member of the Bcl-2 family. Boo was exclusively expressed in the ovary and the epididymis. Boo inhibited apoptosis and interacted with selective pro- and anti-apoptotic Bcl-2 family proteins. Furthermore, Boo bound to Apaf-1 at multiple sites implicating it in the regulation of this central component of the death pathway.

Results

Isolation of Boo, a novel Bcl-2-related gene

In an attempt to identify novel apoptosis-regulatory proteins, we searched the GenBank[TM] expressed-sequence tag (EST) database for cDNAs encoding proteins with homology to chicken neuroretina (NR-13). Several overlapping clones were identified as having statistically significant homology to NR-13. A clone AA623872 (IMAGE Consortium Clone ID 1108004) was characterized further and analysis of its nucleotide sequence revealed an open reading frame that encoded a protein of 191 amino acids with a predicted relative molecular mass of 22 300 Da (Figure 1A). We designated this gene as *Boo*. Sequence analysis revealed that Boo was a novel member of the Bcl-2 family with conserved BH1 and BH2 domains (Figure 1B). Several residues conserved in the BH domains of NR-13, Bcl-2 and Bcl-x_L are different in the Boo sequence. For example, Boo has substituted Ser88 and Arg144 for Gly and Trp found at the equivalent position in other anti-apoptotic Bcl-2 family members (Figure 1B). Hydrophilicity analysis indicated that the protein has a C-terminal transmembrane region (Figure 1B) which is expected to mediate intracellular membrane localization (Kroemer, 1997). Close inspection of its sequence revealed a stretch of 17 amino acids sharing significant homology with the BH4 domain known to be important for the anti-apoptotic function of mammalian Bcl-2 proteins (Figure 1B). However, Boo lacks a recognizable BH3 domain found in all pro-apoptotic members of the Bcl-2 family.

Boo displays a highly restricted expression in mouse tissues

To characterize Boo further, its expression in various mouse adult and embryonic tissues was examined by Northern blot analysis. Surprisingly, Boo mRNA was undetectable or at very low levels in heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis (data not shown). Moreover, no expression was found during embryonic development from E7–E17 (data not shown). Given that all the EST clones were derived from unfertilized, fertilized and 2-cell stage mouse eggs, we reasoned that the expression of Boo may be restricted to the ovary and other reproductive organs. Indeed, further analysis of a mouse RNA master blot, which contains poly(A)$^+$ RNAs from different tissues and developmental stages, showed that Boo mRNA was predominantly expressed in the ovary and weakly in the epididymis but not in the other tissues examined (Figure 2).

Boo is a non-nuclear intracellular protein

To assess subcellular localization, we expressed Boo in 293T cells as a fusion protein with an N-terminal Flag epitope tag. Immuno-staining with anti-Flag antibodies and visualization by fluorescence microscopy was carried out 24 h after transfection. Analysis of labeled cells with an anti-Flag monoclonal antibody revealed that Flag-Boo displayed a compact, granular and extranuclear staining pattern, consistent with an association with membranes of intracellular organelles and the perinuclear region (Figure 3A). The pattern of Boo staining was strikingly similar to that reported for Ced-9, Bcl-2 and Bcl-x_L (Krajewski *et al.*, 1993; Gonzalez-Garcia *et al.*, 1994; Wu *et al.*, 1997b), suggesting that Boo, Bcl-2 and Bcl-x_L localize to similar intracellular compartments in mammalian cells. The specificity of the labeling was confirmed by comparing the staining pattern with that of cells transfected with a control Flag-tagged GATA-1 expression plasmid. Staining of GATA-1 protein with an anti-Flag antibody revealed the nuclear labeling pattern expected for a transcription factor (Figure 3B).

Localization was also assayed independently by subcellular fractionation studies. Transfected 293T cells were

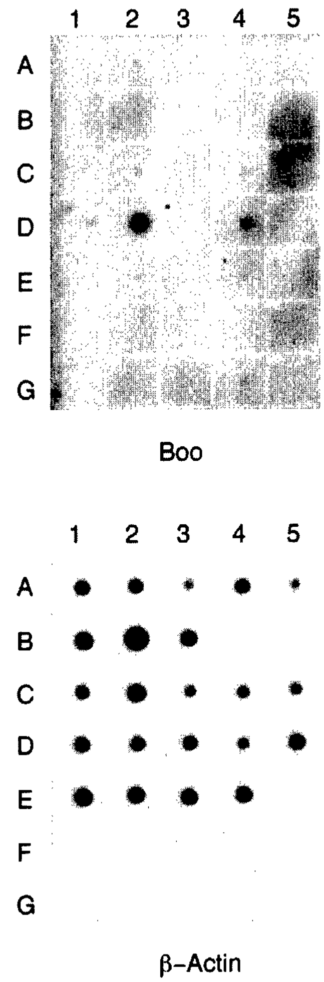

Fig. 2. Distribution of Boo in mouse tissues. Boo was hybridized to poly(A)$^+$ RNA derived from the following mouse tissues: brain (A1), eye (A2), liver (A3), lung (A4), kidney (A5), heart (B1), skeletal muscle (B2), smooth muscle (B3), pancreas (C1), thyroid (C2), thymus (C3), submaxillary gland (C4), spleen (C5), testis (D1), ovary (D2), prostate (D3), epididymis (D4), uterus (D5), embryo 7 days (E1), embryo 11 days (E2), embryo 15 days (E3), embryo 17 days (E4), yeast total RNA (F1), yeast tRNA (F2), *E.coli* rRNA (F3), *E.coli* DNA (F4), poly r(A) (G1), C_0t1 DNA (G2), mouse DNA (G3) and mouse DNA (G4). The same filter was reprobed with β-actin for calibration.

lysed and fractionated into a nuclear, cytosolic and a membrane fraction. Boo was found predominantly in the membrane fraction and some in the low-speed nuclear pellet (Figure 3C). The transcription factor GATA-1 was found exclusively in the nuclear pellet (Figure 3D). These results are consistent with the immunolocalization data and further establish the similarities in subcellular localization between Boo and Bcl-2 (O'Connor *et al.*, 1998).

Overexpression of Boo inhibits apoptosis

To assess its role in regulation of apoptosis, the Flag-tagged Boo expression plasmid was transfected into FL5.12, an interleukin-3 (IL-3)-dependent prolymphocytic cell line and Baf-3, an IL-3-dependent bone marrow-derived cell line. For comparison, cell lines were also transfected with Flag-Bcl-2 and Flag-Bcl-x_L expression plasmids, respectively. Stable clones expressing comparably high

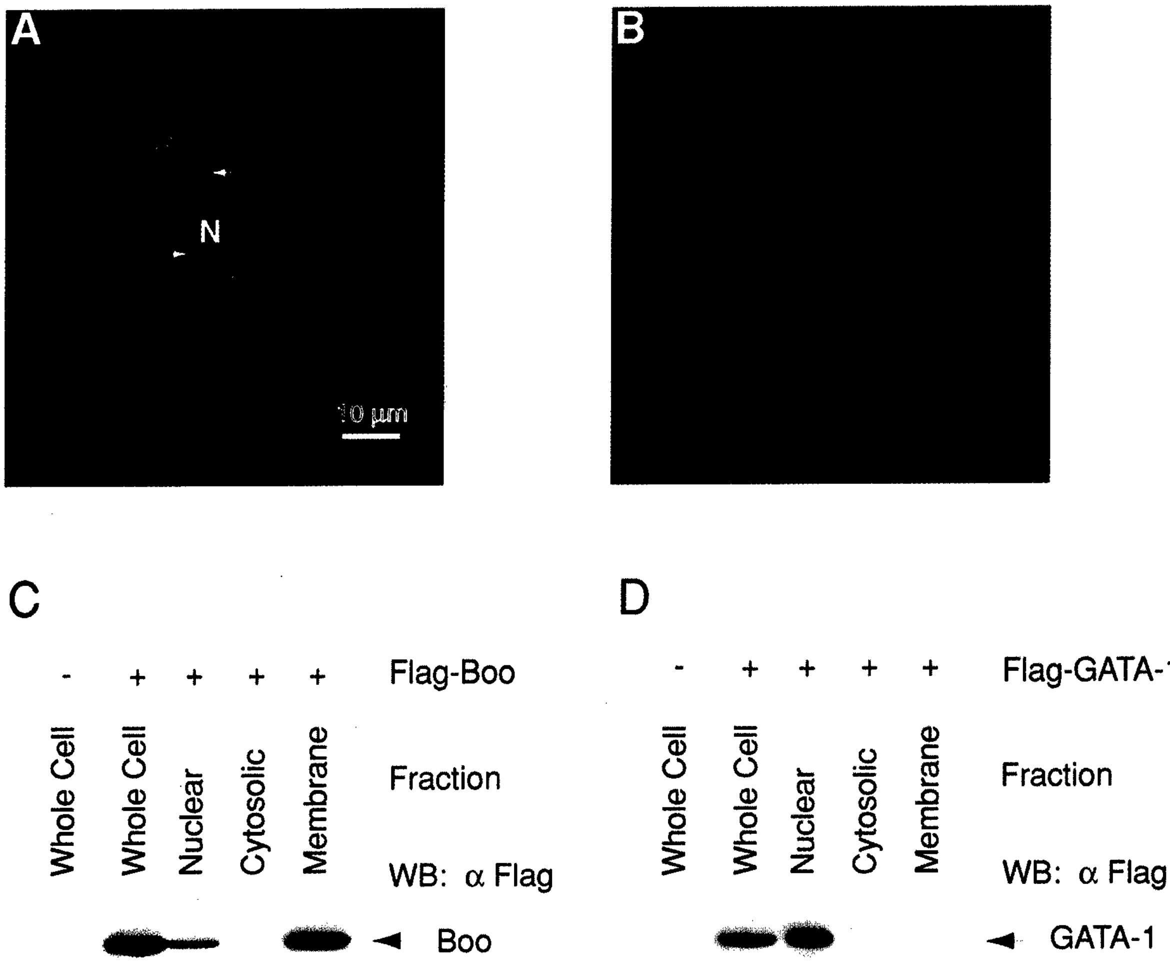

Fig. 3. Subcellular localization of Boo in 293T cells. Cells were transiently transfected with expression plasmids encoding Flag-Boo (**A**) or Flag-GATA-1 as a control (**B**). Shown are images after labeling with anti-Flag and secondary fluorescein-conjugated antibody. Samples were prepared at 24 h after transfection. Arrow, perinuclear region; N, nucleus. Subcellular fractionation of lysates from 293T cells expressing Flag-Boo (**C**) or Flag-GATA-1 (**D**). Lysates from equivalent numbers of unfractionated cells (whole) and of subcellular fractions (nuclear, cytoplasmic or membrane), were resolved by SDS–PAGE and immunoblotted using anti-Flag monoclonal antibody.

levels of Boo, Bcl-2 and Bcl-x_L proteins were selected for further study.

To ascertain whether Boo enhanced or antagonized cell survival, the transfected cell lines were subjected to various cytotoxic conditions. Cells transfected with expression vectors containing only the drug resistance gene served as controls. As shown in Figure 4, both FL5.12 and Baf-3 cells transfected with the neomycin construct alone died rapidly upon IL-3 withdrawal. In contrast, when Boo transfectants were subjected to IL-3 deprivation, they displayed dramatic resistance to cell death and the kinetics of their survival were comparable with that of cells overexpressing either Bcl-2 or Bcl-x_L (Figure 4A and B, left panels). To further compare the anti-apoptotic spectrum of Boo with its homologues, we also exposed FL5.12 and Baf-3 clones to γ-irradiation and cyclosporin A, respectively. Anti-apoptotic Bcl-2 family members have been shown to protect effectively against ionizing radiation-induced cell death as well as the immunosuppressant cyclosporin A (Huang *et al.*, 1997). Boo effectively antagonized both γ-irradiation and cyclosporin A-induced apoptosis (Figure 4A and B, right panels). No significant difference could be detected in the level of protection against these two cytotoxic agents conferred by Boo, Bcl-2 and Bcl-x_L. Collectively, these results clearly place Boo in the sub-family of Bcl-2-related proteins that inhibit cell death.

Localization of mouse Boo gene

Fluorescence *in situ* hybridization analysis using a mouse *Boo* genomic probe clearly assigned *Boo* to mouse chromosome 9 at band d9 (Figure 5). Of the 80 metaphases scored for fluorescent signal using the intron probe, 73 exhibited specific labeling at 9d9.

Boo forms homodimers and heterodimerizes with other Bcl-2 members

To determine whether Boo could form homodimers, we co-transfected 293T cells with expression plasmids producing Flag-Boo and Boo-Myc or a control plasmid. Immunoprecipitates were prepared using an anti-Flag monoclonal antibody, and separated by SDS–PAGE. Immunoblotting with anti-Myc antibody revealed co-precipitated Boo-Myc, indicating the presence of Boo homodimers (Figure 6A). To assess whether Boo interacts with Bcl-2 and Bcl-x_L in mammalian cells, we performed co-immunoprecipitation in 293T cells. As shown in Figure 6B, Boo bound strongly to Bcl-x_L and weakly to

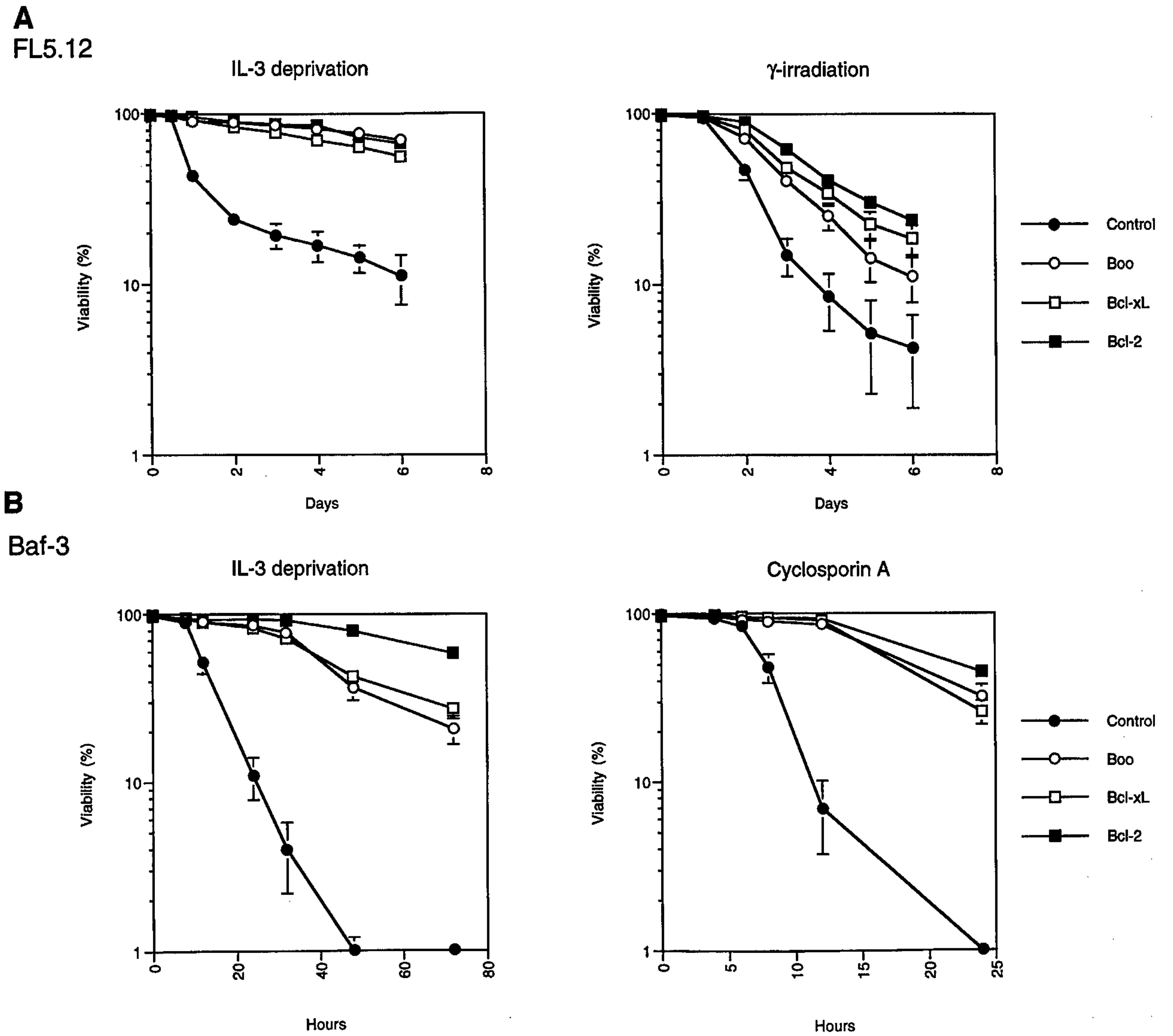

Fig. 4. Effect of Boo, Bcl-2 and Bcl-x_L on apoptosis induced by IL-3 deprivation, γ-irradiation or cyclosporin A. (**A**) FL5.12 cells were either washed three times and cultured in medium lacking IL-3 (left panel) or irradiated (15 Gy) and cultured in conditioned medium (right panel). (**B**) Baf-3 cells were washed three times and cultured in medium lacking IL-3 (left panel) or cultured in non-conditioned medium containing 100 μg/ml cyclosporin A (right panel). Cultures were initiated at 2.5×10^5 cells/ml and cell viability was determined by Trypan blue exclusion. Results are arithmetic means ± SD of at least three experiments and are representative of the results obtained with at least three independent clones.

Bcl-2. Because Boo could inhibit apoptosis induced by different stimuli, we further tested the ability of Boo to interact with Bax, Bak, Bad, Bik and Bid, five pro-apoptotic members of the Bcl-2 family. Associations of Boo with Bak and Bik were readily detected when these proteins were co-expressed *in vivo* (Figure 6C), whereas Bax, Bad and Bid failed to co-precipitate with Boo. These results indicate that Boo interacts only with selective anti- and pro-apoptotic Bcl-2 proteins.

Boo blocks Bak- and Bik-induced apoptosis

In view of the ability of Boo to heterodimerize with Bak and Bik, we next determined whether Boo could block the killing activity of Bak and Bik. In MCF7 cells, overexpression of Bak or Bik induced apoptosis in ~90 and 85% of cells, respectively, but Boo effectively countered Bak- and Bik-mediated killing in this transient assay (Figure 7). On the other hand, Boo did not inhibit Bax-induced cell death (Figure 7). Thus, the anti-apoptotic activity of Boo correlated with its ability to heterodimerize to pro-apoptotic Bcl-2 homologues.

Boo interacts at multiple sites with Apaf-1

Recent reports demonstrate that Bcl-x_L can interact with Apaf-1, the mammalian counterpart of Ced-4 (Hu *et al.*, 1998; Pan *et al.*, 1998). We speculated that an equivalent interaction might exist between Boo and Apaf-1. To test this, 293T cells were transiently co-transfected with Apaf-1-Myc and Boo-Flag or Flag-A20, a control protein. Western blot analysis of protein complexes immunoprecipitated with anti-Flag antibody revealed that Apaf-1-Myc co-precipitated with Boo-Flag, but not Flag-A20 (Figure 8B). To determine the interaction specificity between Boo and Apaf-1, the immunoprecipitation was also performed in reverse using anti-Myc monoclonal antibody. As shown in Figure 8, Boo-Flag but not Flag-A20 co-precipitated with Apaf-1-Myc. To define critical regions in Apaf-1 required for its interaction with Boo,

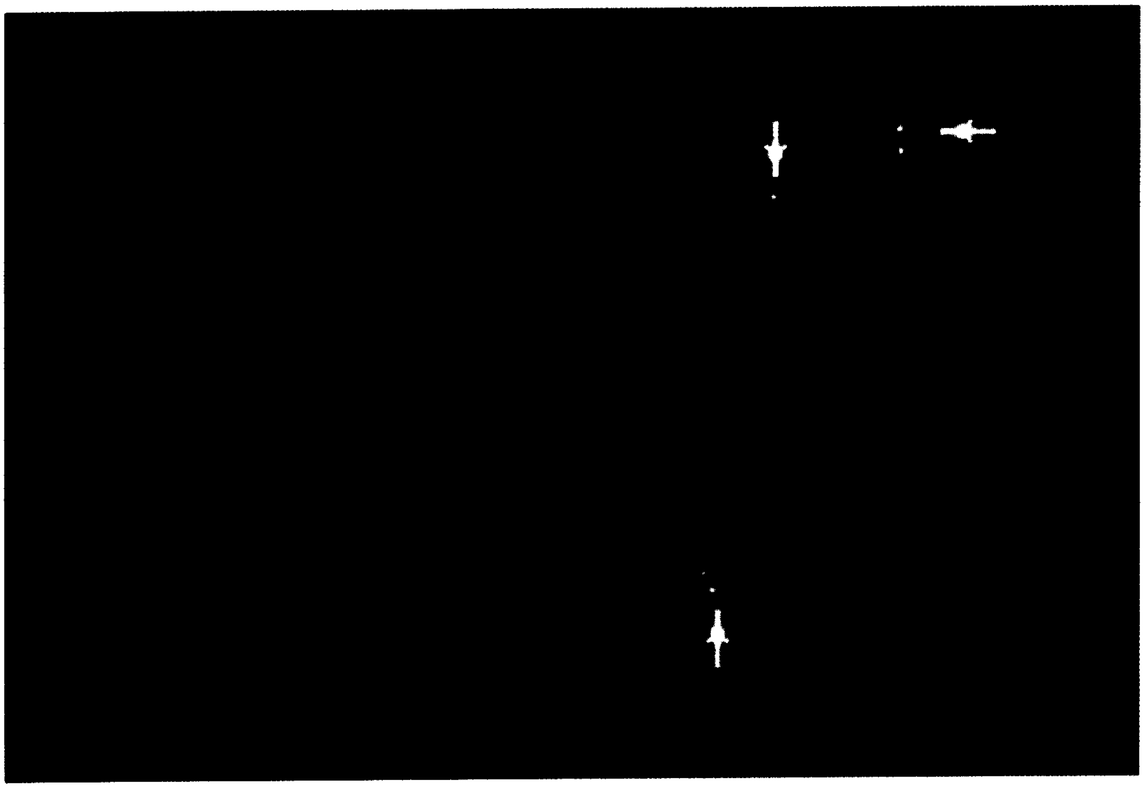

Fig. 5. Localization of Boo on mouse chromosome 9. A Boo genomic probe was used to hybridize to normal metaphase chromosomes derived from mouse embryo fibroblast cells. Hybridization sites on chromosome 9 are indicated by arrows.

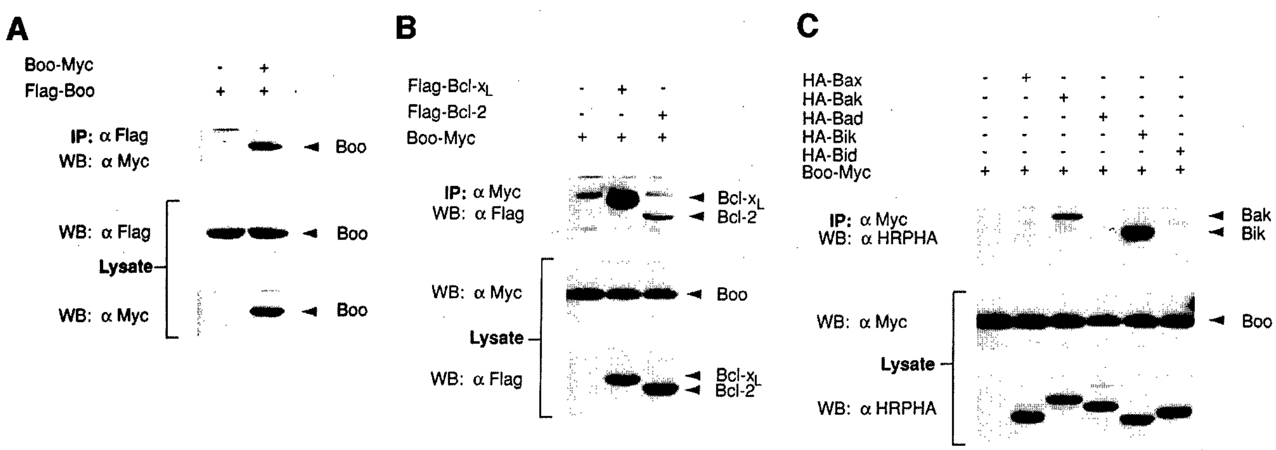

Fig. 6. Boo forms homodimers and interacts with selective Bcl-2 family proteins. (A) Homodimerization. 293T cells were transiently transfected with Flag-Boo and Boo-Myc or a control vector. Cell lysates were prepared and immunoprecipitated as described in Materials and methods with the indicated antibodies. Lysates and immunoprecipitates were analyzed by Western blotting as shown. (B) Heterodimerization with Bcl-x_L and Bcl-2. 293T cells were transfected with epitope-tagged constructs, and lysates and immunoprecipitates were analyzed by Western blotting as indicated. (C) Boo selectively binds to Bak and Bik. Cells were co-transfected with Boo-Myc plus pro-apoptotic Bcl-2 family members. Analysis of lysates and immunoprecipitates was performed by Western blotting as indicated. WB, Western blot analysis; IP, immunoprecipitation.

we engineered five deletion mutants of Apaf-1 (Figure 8A). In initial experiments, an N-terminal deletion mutant (N-Apaf-1; residues 1–601), that lacks the WD-40 repeat region and a C-terminal deletion mutant (C-Apaf-1; residues 602–1194), that contains only the WD-40 repeat domain were assessed for their ability to interact with Boo in a co-transfection assay. Western blot analysis of Boo complexes with anti-Myc antibody revealed that both the N-Apaf-1 and C-Apaf-1 mutants associated with Boo (Figure 8B). Furthermore, Boo also bound to a truncated form of Apaf-1 [Apaf-1(3+4)], that comprises the Ced-3 and Ced-4 homologous regions, and the Apaf-1 unique segment between the Ced-4-like and WD-40 repeat domains (Figure 8B). However, Apaf-1(3), a deletion mutant containing the Ced-3-homologous region, failed to co-precipitate with Boo (Figure 8B). Thus, these results indicate that Boo binds to at least three distinct domains in Apaf-1, the Ced-4-homologous region, the WD-40 repeats, and the Apaf-1 unique domain between the Ced-4-like and WD-40 repeat regions.

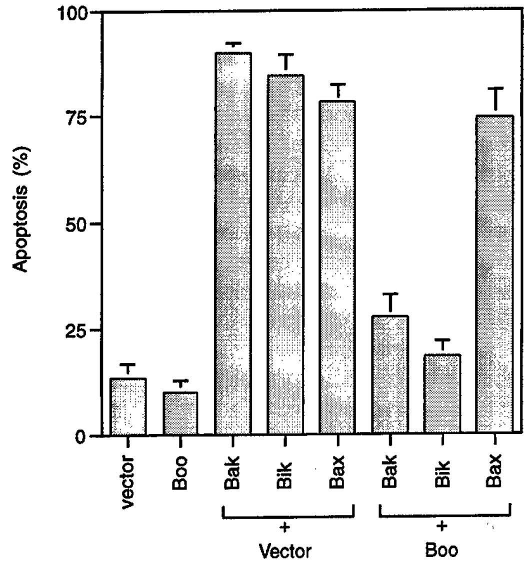

Fig. 7. Boo inhibits apoptosis induced by Bak or Bik. MCF7 cells were co-transfected with Bak, Bik or Bax together with a β-galactosidase-expressing reporter construct in the presence of a 4-fold excess of a control vector or Boo. Cells were stained and examined as described (Duan *et al.*, 1996).

Boo and caspase-9 form a ternary complex with Apaf-1

Given that Bcl-x_L and caspase-9 form a ternary complex with Apaf-1 (Pan *et al.*, 1998), we investigated whether an analogous biochemical interaction may also occur with Boo. To assess this, 293T cells were transiently co-transfected with Boo-Myc and caspase-9–DN-Flag (DN, dominant negative). Immunoprecipitation of caspase-9–DN-Flag coprecipitated Boo-Myc, but not Myc-TRADD, a control protein (Figure 9A). A recent study has shown that the Ced-3 homologous domain of Apaf-1 interacts with caspase-9 but not Bcl-x_L (Pan *et al.*, 1998). Similarly, in the present study, this truncated version of Apaf-1 also failed to bind to Boo. To determine further whether the *in vivo* interaction between caspase-9 and Boo was mediated by an endogenous Apaf-1-like protein, expression plasmids producing caspase-9–DN-Flag, Boo-Myc and Apaf-1(3)-Myc were co-transfected in 293T cells. Expression of Apaf-1(3)-Myc in excess significantly attenuated the ability of caspase-9 to coprecipitate Boo (Figure 9B), suggesting that this deletion mutant of Apaf-1 competitively blocked the interaction of caspase-9 with an endogenous Apaf-1-like molecule, thereby disrupting the association between caspase-9 and Boo. Moreover, ectopic expression of Boo did not affect the binding of caspase-9 to Apaf-1 (Figure 9C), further confirming that Apaf-1 simultaneously interacts with caspase-9 and Boo, forming a ternary complex in mammalian cells. The ability of Bak and Bik to heterodimerize with Boo prompted us to examine whether these two pro-apoptotic members of the Bcl-2 family may function by affecting the association of Boo with Apaf-1. Co-expression of hemagglutinin (HA)-Bak or HA-Bik disrupted the interaction between Boo and Apaf-1 (Figure 9D), indicating that both death promoters may exert their killing activity by abrogating the recruitment of Boo to Apaf-1 and thus favoring Apaf-1-mediated caspase-9 activation.

Discussion

We have identified a novel regulator of apoptosis, Boo, which is homologous to Bcl-2, the prototype of the Bcl-2 family. When stably expressed, Boo greatly enhances the survival of cells exposed to growth factor deprivation, γ-irradiation or an immunosuppressant, cyclosporin A. This wide spectrum of anti-apoptotic activity is characteristic of the Bcl-2 family (Reed, 1994; Huang *et al.*, 1997). Boo is also protective in transient assays and effectively inhibits Bik and Bak induced apoptosis. We therefore conclude that Boo, Bcl-2, and Bcl-x_L are functional homologues. Boo clearly falls into the death-repressing rather than the death-promoting sub-group of the Bcl-2 family.

Among the anti-apoptotic Bcl-2 proteins, Boo is most similar to chicken NR-13. Alignment analysis reveals that the Boo protein contains conserved BH1 and BH2 domains, but lacks the BH3 motif found in all the death agonists. The presence of a C-terminal transmembrane region is expected to mediate localization of Boo to intracellular membranes. Immunofluorescence and subcellular localization experiments in 293T cells support this view.

Notably, Boo has a serine at position 88 and an arginine at 144. These residues corresponding to G142 in the BH1 and W185 in the BH2 domain of Bcl-2, which are highly conserved among other anti-apoptotic family proteins (Yin *et al.*, 1994; Sedlak *et al.*, 1995). Previous studies have shown that these positions are important for Bcl-2 function and heterodimerization with Bax (Yin *et al.*, 1994). However, replacement of the W residue in Bcl-x_L had minimal effect on their survival function (Cheng *et al.*, 1996). Moreover, substitution of the G residue with an E residue in Ced-9 resulted in a gain-of-function mutant (Hengartner and Horvitz, 1994a). These results indicate that the residues necessary for the pro-survival activity of Bcl-2 are not identical to those of Ced-9 and Bcl-x_L. We mutated these positions in Boo and found that expression of the mutant Boo was equally effective at inhibiting Bak and Bik induced apoptosis (unpublished data). Thus, Boo is more similar to Ced-9 and Bcl-x_L in its ability to incorporate silent mutations at these positions.

Recent studies have shown that deletion of the BH4 domain rendered Bcl-2 and Bcl-x_L inactive (Huang *et al.*, 1998), indicating that this domain is essential for death-suppressing function of Bcl-2 and Bcl-x_L. Consistent with this finding, Boo also has a recognizable BH4 domain, which may contribute to its inhibitory activity.

The nuclear magnetic resonance (NMR) structural analysis of Bcl-x_L has shown that its BH1, BH2 and BH3 domains form an elongated hydrophobic cleft which can bind the BH3 domains of death-promoting proteins (Sattler *et al.*, 1997). Since Boo lacks a BH3 motif, it probably has an interaction-interface distinct from that of Bcl-x_L. In agreement with this hypothesis, Boo shows a selective heterodimerization profile by interacting with some (Bak and Bik) but not other (Bax, Bad and Bid) pro-apoptotic members. Similarly, Bok/Mtd, a pro-apoptotic Bcl-2 homologue, which lacks a BH4 domain and has a less conserved BH3 domain than other Bcl-2 proteins, interacts only with selective anti-apoptotic family molecules (Mcl-1, BHRF1 and Bfl-1) (Hsu *et al.*, 1997; Inohara *et al.*, 1998a).

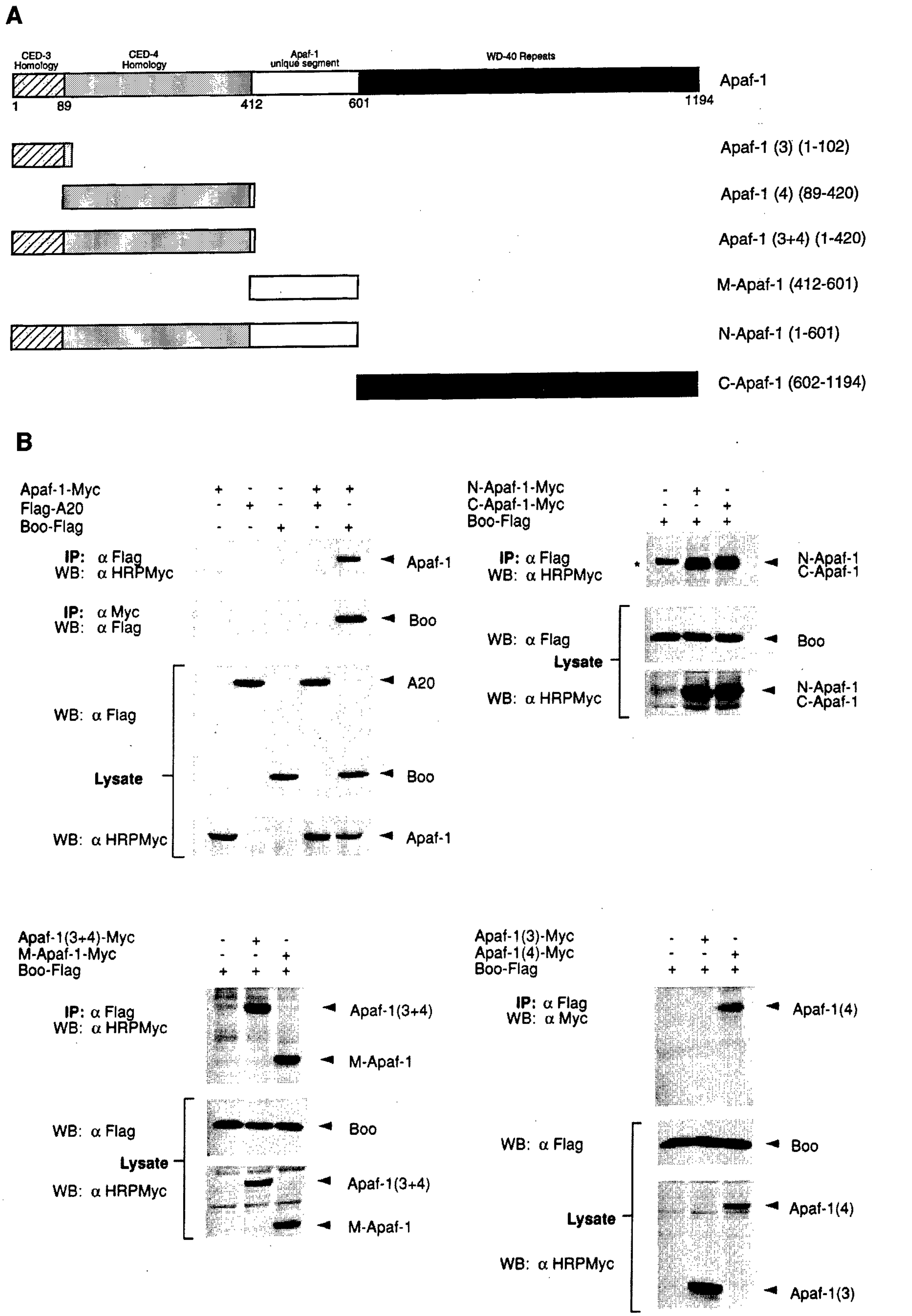

Fig. 8. Boo binds to distinct domains in Apaf-1. (**A**) Schematic drawing of the Apaf-1 constructs used in this study. (**B**) 293T cells were co-transfected with Boo-Flag and the indicated Apaf-1 constructs or a control vector. Flag or Myc immunoprecipitates were analyzed for co-precipitating proteins by immunoblotting with mAb towards Myc, Flag or horseradish peroxidase (HRP)-conjugated anti-Myc. Expression of Boo and the Apaf-1 constructs was visualized by Western blotting of the lysates with αFlag or αHRP-Myc antibodies. Asterisk indicates a non-specific band. WB, Western blot analysis; IP, immunoprecipitation.

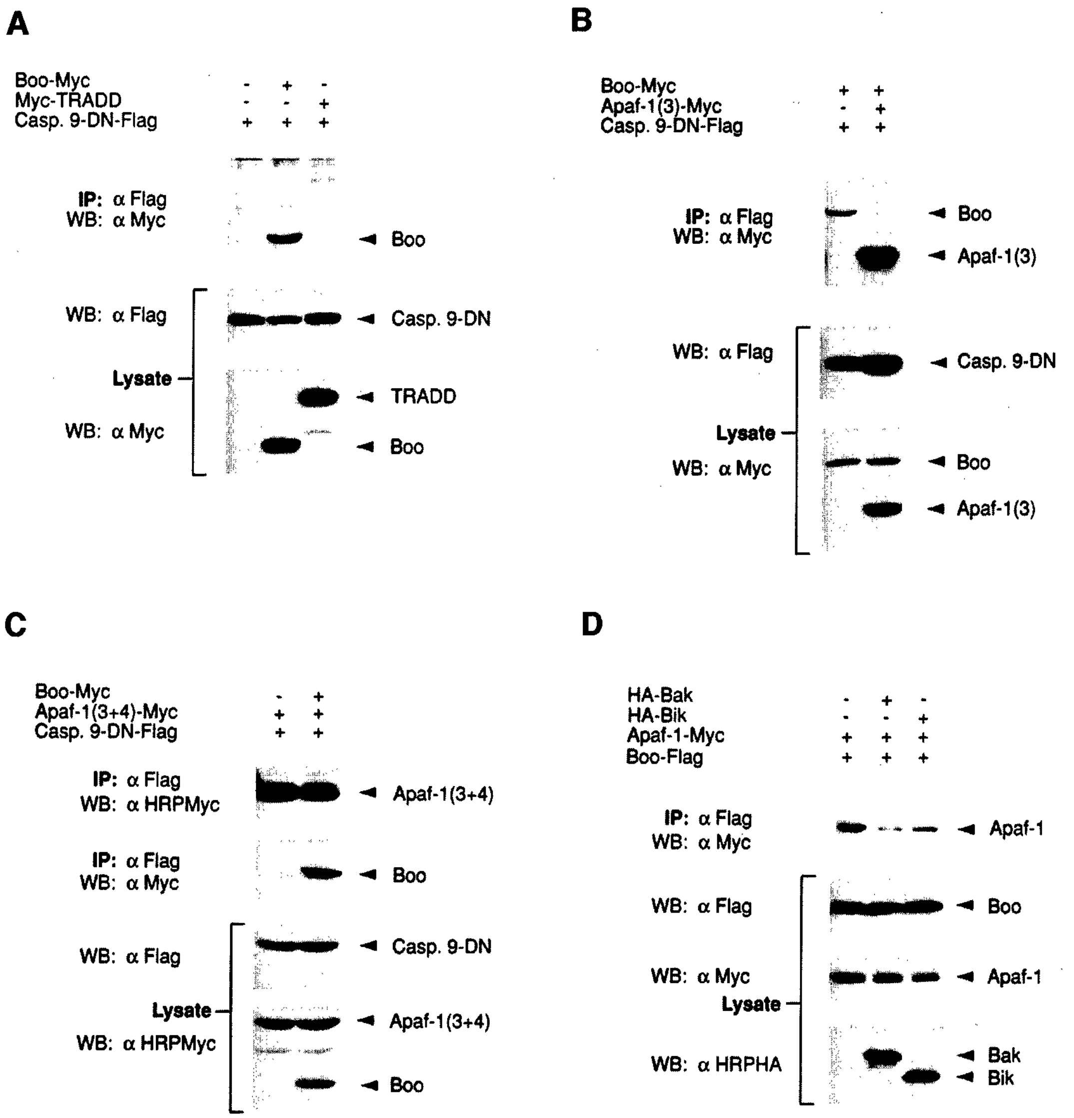

Fig. 9. Boo and caspase-9 form a multimeric complex with Apaf-1. (**A**) Caspase-9 associates with Boo through an endogenous Apaf-1-like molecule. 293T cells were co-transfected with caspase-9–DN-Flag and Boo-Myc, Myc-TRADD or a control vector. Anti-Flag immunoprecipitates were blotted with anti-Myc. Expression of the transfected gene products was assayed in lysates by Western blotting as indicated. (**B**) Co-expression of the Ced-3-homologous region of Apaf-1 disrupts the association of caspase-9 with Boo. 239T cells were co-transfected with caspase-9–DN-Flag and Boo-Myc in the presence of a control vector or a construct expressing Apaf-1(3)-Myc. Immunoprecipitation and Western blot analysis was performed as in (A). (**C**) Boo does not affect the interaction between caspase-9 and Apaf-1. Caspase-9–DN-Flag was co-expressed with Apaf-1(3+4)-Myc in the presence of either a control vector or a Boo-Myc construct. Immunoprecipitation was carried out with anti-Flag affinity gel and blotted with anti-Myc or HRP-conjugated anti-Myc. Expression of the transfected gene products was assayed in lysates by Western blotting as indicated. (**D**) Bak or Bik disrupts the interaction between Apaf-1 and Boo. Boo-Flag and Apaf-1-Myc were co-expressed in the presence of either a control vector, HA-Bak or HA-Bik. Anti-Flag immunoprecipitates were blotted with anti-Myc. Lysates were analyzed for expression of the transfected gene products by Western blotting with anti-epitope antibodies as shown. WB, Western blot analysis; IP, immunoprecipitation.

Moreover, the anti-apoptotic KS-Bcl-2 protein, which contains a poorly conserved BH3 domain and lacks a BH4 region, can bind neither to Bak and Bik nor to Bcl-2 and Bcl-x_L (Cheng *et al.*, 1997). These observations suggest that anti-apoptotic proteins have a variety of interaction surfaces for Bcl-2 homologues, and that the binding specificity is determined by multiple domains. However, only detailed structural information will permit full understanding of these protein–protein interactions. Furthermore, restricted heterodimerization between pro- and anti-survival members of the family may have evolved to regulate multiple death and survival signals.

Interestingly, Boo can also interact with Bcl-2 and Bcl-x_L. Consistent with this observation, other death antagonists such as Bcl-x_L, Mcl-1 and BHRF1 have been shown to interact with Bcl-2 (Farrow and Brown, 1996; Brown, 1997). However, the precise role of these dimerizations between pro-survival proteins in regulation of apoptosis remains unclear.

It has been proposed that the ratio of pro- to anti-apoptotic members and their dimerization determine death or survival. For example, Bcl-2 mutants fails to heterodimerize with Bax and no longer can inhibit apoptosis (Yin *et al.*, 1994). However, this view was challenged by the identification of Bcl-x_L mutants and a viral homologue, KS-Bcl-2, that do not interact with Bak or Bik but still inhibit apoptosis (Cheng *et al.*, 1996, 1997). Surprisingly, a recent study has shown that Bcl-2 and Bcl-x_L mutants that can not heterodimerize with Bax or Bak still retain the ability to interact with Bad (Ottilie *et al.*, 1997), suggesting that it is difficult to draw definite conclusions from these studies because of the redundancy among Bcl-2 family proteins.

How might Boo function given that it interacts only with a subset of Bcl-2 death agonist but protects from a broad range of apoptotic stimuli? The recent discovery that Apaf-1 can form a ternary complex with caspase-9 and Bcl-x_L has raised the possibility that the death-suppressing members of the Bcl-2 family function by regulating Apaf-1-like proteins and thereby controlling activation of procaspases and cell death (Li *et al.*, 1997; Hu *et al.*, 1998; Pan *et al.*, 1998). In keeping with this hypothesis, here we have shown that Boo interacts with Apaf-1. We further demonstrate that Boo associates with caspase-9. The biochemical linkage between Boo and caspase-9 could be attenuated by the presence of a deletion mutant of Apaf-1 that binds caspase-9 but not Boo. This led to the suggestion that the interaction between Boo and caspase-9 is mediated by an endogenous Apaf-1 and that mutant Apaf-1 blocks the recruitment of endogenous Apaf-1 and thus Boo to caspase-9. The observation that overexpressed Boo did not compete for the association between caspase-9 and Apaf-1 provides additional evidence that Apaf-1 simultaneously interacts with caspase-9 and Boo, forming a ternary complex in mammalian cells. This complex can be disrupted by Bak and Bik. These results, taken together with the recent evidence that Bax and Bak can abolish the association of Bcl-x_L with Apaf-1 (Pan *et al.*, 1998), indicate that the death-promoting Bcl-2 family homologues may function by binding to death-repressing members of the Bcl-2 family and displacing Apaf-1-like molecules, thereby permitting the activation of procaspases. These data imply that anti-apoptotic Bcl-2 homologues have the dominant function in regulating apoptosis.

We found that Boo binds to three distinct regions in Apaf-1. Since Bcl-x_L could not inhibit caspase-9 maturation mediated by an N-terminal mutant of Apaf-1 which contains only the Ced-4-like domain (Hu *et al.*, 1998), it is conceivable that both the WD-40 repeat region and the Apaf-1 unique segment act as regulatory elements controlling the activity of Apaf-1. Binding of Boo to these two domains could induce a conformational change in Apaf-1 that would inactivate Apaf-1 and thus inhibit the processing of caspase-9. We are currently testing these possibilities.

The tissue distribution of Boo is the most restricted of any of the Bcl-2 family members. The expression of Boo in the epipidymis may indicate that it contributes to sperm maturation. A precedent for a specific role of the Bcl-2 family of proteins in the regulation of the male reproductive tract is the ROSA41 mouse strain that carries a disrupted *Bclw* gene. Although *Bclw* is expressed widely, its inactivation leads mainly to reduced size of the testis and seminal vesicles causing sterility (Ross *et al.*, 1998). Bcl-2 overexpression in spermatogonia and loss of function of Bax also leads to disruption of spermatogenesis (Knudson *et al.*, 1995; Furuchi *et al.*, 1996). This implies that several Bcl-2 homologues with very similar functional properties are necessary during different stages of sperm maturation and Boo is likely to be important during the epipididymal phase.

Highest expression of Boo is detected in the ovaries. Cell loss in the ovaries occurs during pre and post-natal ovarian development (Beaumont and Mandl, 1962). Apoptotic cell death plays an important role during this process (Tilly, 1996). In addition, oocytes are particularly sensitive to chemo- and radiotherapy and such treatments often lead to sterility (Familiari *et al.*, 1993; Ried and Jaffe, 1994). Therefore, understanding the molecular processes specific for ovarian apoptosis are of considerable interest. Boo is not the only anti-apoptotic Bcl-2 homologue expressed in the ovary. Mcl-1, Bcl-2 and Bcl-x have also been detected in ovarian tissues (Tilly *et al.*, 1995; Hsu *et al.*, 1997). Multiple pro- and anti-apoptotic proteins have been shown to have an effect on oocyte apoptosis. For example, Bcl-2 knock-out mice contain aberrantly formed primordial follicles (Ratts *et al.*, 1995). The oocytes of Bax as well as caspase-2 knock-out mice are remarkably resistant to doxorubicin-induced apoptosis (Perez *et al.*, 1997; Bergeron *et al.*, 1998). Therefore, more detailed studies need to be carried out to understand the interplay of these molecules in the ovarian context. Targeted gene disruption of *Boo* should be part of such an analysis.

Materials and methods

Cloning Boo and expression vectors

The partial nucleotide sequences of cDNAs encoding peptides with homology to chicken NR-13 were found in EST database of GenBank™ using the TBLASTN program. The nucleotide sequence of EST clones 1108004 (DDBJ/EMBL/GenBank accession No. AA623872) was determined by dideoxy sequencing. The full-length Boo from EST clone 1108004 was cloned into pcDNA3 (Invitrogen) with a N- or C-terminal Flag tag, or a C-terminal Myc tag. The full-length Apaf-1 was cloned into pcDNA3.1(–)/Myc-His B (Invitrogen). The truncated mutants of Apaf-1, N-Apaf-1, C-Apaf-1, M-Apaf-1, Apaf-1(3+4), Apaf-1(3) and Apaf-1(4) were amplified by PCR using the Apaf-1 cDNA as a template and also cloned into pcDNA3.1(–)/Myc-His B. Bad and Bid were cloned into pcDNA3 with a N-terminal HA tag. The constructs encoding Flag-Bcl-2, Flag-Bcl-x_L, HA-Bax, HA-Bak, HA-Bik, caspase-9–DN-Flag and Myc-TRADD have been described previously (Chinnaiyan *et al.*, 1996, 1997; Duan *et al.*, 1996; Duan and Dixit, 1997).

Northern blot analysis

A fragment (nucleotides 1–1008) of the Boo cDNA was radiolabeled by PCR-labeling method and applied for analysis of mouse adult and embryo multiple-tissue Northern blots, and mouse RNA master blot (Clontech) according to the manufacturer's instructions. The mouse RNA master blot was also hybridized with a β-actin cDNA probe.

Tissue culture, stable transfection and cell-viability assay

MCF7, a human breast carcinoma cell line was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), non-essential amino acids, L-glutamine, and penicillin/streptomycin. FL5.12, an IL-3-dependent murine pro-B lymphocyte line and Baf-3, an IL-3-dependent bone marrow-derived line were cultured in the same medium supplemented with 10% WEHI-3B-conditioned medium as a source of IL-3. Dulbecco's modified Eagle's medium containing 10% FBS, non-essential amino acids, L-glutamine and penicillin/streptomycin was used to maintain the 293T cells.

FL5.12 and Baf-3 cells were transfected using a Gene-pulser (Bio-Rad) with pcDNA3 plasmid containing Flag-Boo, Flag-Bcl-2 or Flag-Bcl-x_L or a control pcDNA3 plasmid (200 V, 960 μF). Transfectants were selected by growth in G418 (1 mg/ml). Individual clones were isolated by limiting dilution of a polyclonal neomycin-resistant population into 96-well plates and then screened for the expression of the transfected gene of interest by immunoblotting. Clones expressing comparably high levels of Flag-Boo, Flag-Bcl-2 and Flag-Bcl-x_L were selected for further study.

To test their sensitivity to apoptosis, FL5.12 cells were cultured in IL-3-free medium, or in IL-3-containing medium after exposure to 15 Gy of γ-irradiation (provided by a ^{60}Co source at a rate of 215 cGy/min). Baf-3 cells were cultured in IL-3-free medium, or in IL-3-free medium supplemented with 100 μg/ml of cyclosporin A (Sigma). Cell viability was determined by Trypan blue exclusion and counting in a hemocytometer. The percentage of survival cells represents the mean value from at least three independent experiments with at least three independent clones.

Immunofluorescence, subcellular fractionation, immunoprecipitation and immunoblotting

To investigate the subcellular localization of Boo, 293T cells were transfected with pcDNA3–Flag-Boo, pcDNA–Flag-GATA-1, or empty vector as described above. Twenty-four hours after transfection, cells were incubated with anti-Flag antibody for 1 h at 23°C and the labeling was visualized with fluorescein-conjugated goat anti-mouse IgG. After washing, the cells were mounted in Vectashield mounting medium (Vector Laboratories, Inc.) and examined with a Leitz orthoplan fluorescence microscope. To test for protein–protein interactions *in vivo*, 293T cells were transiently transfected with indicated plasmids by calcium phosphate precipitation.

For subcellular fractionation, lysates were prepared as described previously (O'Connor *et al.*, 1998). Briefly, 2×10^6 cells were homogenized in 1 ml of hypotonic lysis buffer (10 mM Tris–HCl pH 7.4, 0.5 μg/ml Pefabloc, 1 μg/ml each of leupeptin, aprotinin, soybean trypsin inhibitor and pepstatin, 5 mM NaF and 2 mM Na_3VO_4) with a Dounce homogenizer. The lysates were centrifuged at 900 *g* for 10 min to obtain the nuclear pellet and the supernatant centrifuged at 130 000 *g* for 60 min to obtain the soluble cytosolic and the pelleted membrane fractions.

Protein immunoprecipitation and Western blot analysis with relevant antibodies were performed as described previously (Duan and Dixit, 1997). Briefly, cells were lysed in 1 ml of lysis buffer (20 mM Tris pH 8.0, 137 mM NaCI, 10% glycerol, 1% nonident P40, 2 mM EDTA) 24–48 h after transfection and soluble lysates were incubated with anti-Flag, anti-Myc or anti-HA antibodies and protein G–Sepharose (Sigma) overnight at 4°C. Immune complexes were centrifuged, washed with excess cold lysis buffer at least three times, and then boiled in gel-loading buffer. Eluted proteins were subjected to SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-Flag, -Myc or -HA antibodies.

Apoptosis assay

These were performed essentially as described previously (Duan *et al.*, 1996). Briefly, MCF7 cells were plated on a six-well tissue culture plate (2×10^5 cells/well) and transiently transfected with 0.1 μg of the reporter plasmid pCMV-β-galactosidase plus 0.25 μg of pcDNA3–HA-Bak or pcDNA3–HA-Bik in the presence or absence of 1 μg of pcDNA3–Flag-Boo by the Lipofectamine procedure. The total amount of transfected plasmid DNA was adjusted to 1.5 μg/well by adding pcDNA3. Transfected cells were detected by staining with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside as described previously (Duan *et al.*, 1996). The percentage of apoptotic cells represents the mean value from three independent experiments.

Fluorescence in situ hybridization

A mouse Boo genomic probe was labeled with digoxigenin dUTP by nick translation. Labeled probe was combined with sheared mouse DNA and hybridized to normal metaphase chromosomes derived from mouse embryo fibroblast cells in a solution containing 50% formamide, 10% dextran sulfate and 2 × saline-sodium citrate buffer. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated anti-digoxigenin antibodies followed by counterstaining with 4,6-diamidino-2-phenylindole (DAPI).

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Note added in proof

The gene for Boo has been recently described as DIVA (Inohara *et al.*, 1998b).